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APPLICATION

FOR

UNITED STATES LETTERS PATENT

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TITLE : TRANSGENIC PLANTS EXPRESSING A MAPKKK PROTEIN KINASE DOMAIN

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TRANSGENIC PLANTS EXPRESSING A MAPKKK PROTEIN KINASE DOMAIN

Background of the Invention

This application is a continuation of 09/371,338 filed August 10. 1999, and claims the benefit of U.S. provisional application Ser. No. 60/095,938 filed on August 10, 1998.

This invention relates to the manipulation of plant gene expression and the production of transgenic plants.

Auxin is an essential plant hormone that regulates diverse processes, such as cell division and elongation, root and leaf development, apical dominance, tropism, and reproduction (Davies, P. J., *In*: Plant hormones, ed., Davies, P.J., pp. 1-12, Kluwer, Dordrecht, Netherlands, 1995.). The auxin response is regulated by a complex signaling network, and reflects a balance between auxin and other synergistical or antagonistical signaling pathways in plant cells (Bellincampi et al., *Plant Cell* 8: 477-487, 1996; Coenen et al., *Trends Plant Sci.* 2: 351-356, 1997). A primary event of auxin action is the activation of many early response genes. Extensive studies of the early response gene promoters have identified several auxin responsive cis-elements and trans-acting factors (Abel et al., *Plant Physiol.* 111: 9-17, 1996; Ulmasov et al., *Science* 276: 1865-1868, 1997). Although genetic approaches have significantly advanced our understanding of auxin action (Walden et al., *Trends Plant Sci.* 1: 335-339, 1996; Leyser, *Curr. Biol.* 8: R305-R307, 1998; Guilfoyle, *Trends Plant Sci.* 3: 205-207, 1998), the molecular mechanisms underlying signal transduction pathways that control auxin responsive transcription remain largely unknown.

In yeast, worms, insects, and mammals, the primary responses to hormone, growth, and stress signals are mediated by a conserved signaling cascade consisting of three protein kinases, the mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase kinase (MAPKK), and mitogen-activated protein kinase kinase kinase

(MAPKKK). MAPKKK phosphorylates and activates MAPKK that, in turn, phosphorylates and activates MAPK. The activated MAPK can be translocated into the nucleus where it phosphorylates transcription factors that control gene expression (Herskowitz, *Cell* 80: 187-197, 1995; Kyriakis et al., *J. Biol. Chem.* 271: 24313-24316, 1996). Although many plant MAPK, MAPKK, and MAPKKK homologues have been identified based on sequence conservation and functional complementation in yeast, their precise physiological functions in plants are largely unknown (Hirt, *Trends Biol. Sci.* 2: 11-15, 1997). It also remains unclear whether and how these homologues constitute specific MAPK kinase cascades (Mizoguchi et al., *Trends Biotech.* 15: 15-19, 1997).

Plants are constantly exposed to environmental stimuli that influence their growth and development. Adverse environmental conditions, including heat, salinity, freezing, and drought, greatly compromise plant productivity and reduce crop yield. Genetic approaches have been taken to enhance plant tolerance to stresses through alteration of osmolytes, osmoprotectants, membrane fatty acids, channels, transcription factors, and enzymes that scavenge active oxygen species by transferring or mutating individual stress target genes. A need in the art therefore exists for developing molecular strategies that enable plants to have resistance or tolerance to adverse environmental conditions.

Summary of the Invention

The invention is based on applicants' discovery that a mitogen-activated protein kinase kinase kinase (MAPKKK) polypeptide, such as NPK1 of tobacco and the ANPs of *Arabidopsis*, is involved in signaling the activation of stress protective gene transcription, repression of early auxin response gene transcription, and the alteration of seed development. Accordingly, the invention involves methods of genetically engineering plants to produce altered, agronomic, physiological, or developmental changes in plants by expressing a transgene including DNA encoding a kinase domain of

a MAPKKK within the tissues of the plants. In particular, it has been found that it is possible to engineer plants that express a recombinant MAPKKK that are resistant to a broad spectrum of stresses (e.g., drought, increased salinity, heat shock, and freezing temperature), that have repressed early auxin gene expression, or that have altered seed development.

In one aspect, the invention therefore features a method for increasing stress resistance or tolerance in a plant. The method, in general, includes the steps of: (a) introducing into plant cells a transgene including DNA encoding a kinase domain of a MAPKKK operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a transgenic plant from the transformed cells, wherein the kinase domain of the MAPKKK is expressed in the cells of the transgenic plant, thereby increasing the level of stress resistance or tolerance in the transgenic plant. In preferred embodiments, the expression of the DNA encoding the kinase domain activates the expression of a stress-inducible gene (e.g., a gene encoding a glutathione S-transferase, an asparagine synthetase, or a heat shock protein). In particular applications, the method is especially useful for providing to a plant resistance or tolerance to an environmental stress. Exemplary environmental stresses include, without limitation, those which occur upon exposure of the transgenic plant to limited or inadequate water availability (e.g., drought conditions), excess salt or osmotic conditions, excess temperature conditions (e.g., heat, cold, or frost), excess light, a pathogen, a chemical (e.g. a metal, herbicides, and pollutants), an oxidative stress, UV light, and wounding. In preferred embodiments, the plant is protected against multiple stress conditions.

In another aspect, the invention features a method for reducing the action of an auxin in a plant. The method includes the steps of: (a) introducing into plant cells a transgene including DNA encoding a kinase domain of a MAPKKK operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a transgenic plant from the transformed cells, wherein the kinase domain of the MAPKKK

is expressed in the cells of the transgenic plant, thereby reducing the action of the auxin in the transgenic plant. In preferred embodiments, the expression of the DNA encoding the kinase domain represses the expression of an early-auxin gene (e.g., those which are under the control of a promoter which is substantially identical to the GH3 promoter or a promoter which includes the ER7 element).

In still another aspect, the invention features a method for altering seed development. In particular, the method includes the steps of: (a) introducing into plant cells a transgene including DNA encoding a kinase domain of a MAPKKK operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a transgenic plant from the transformed cells, wherein the kinase domain of the MAPKKK is expressed in the cells of the transgenic plant, thereby altering the development of a seed in the transgenic plant. In preferred embodiments, the expression of the DNA encoding the kinase domain enriches endosperm development, enriches embryo development, or attenuates seed development. In yet other preferred embodiments, the attenuation of the seed development results in a seedless plant (e.g., a seedless fruit or vegetable).

In yet another aspect, the invention features a method for increasing the yield or productivity of a transgenic plant. The method generally includes the steps of: (a) introducing into plant cells a transgene including DNA encoding a kinase domain of a MAPKKK operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a transgenic plant from the transformed cells, wherein the kinase domain of the MAPKKK is expressed in the cells of the transgenic plant, thereby increasing the yield of the transgenic plant.

In related aspects of the invention, the invention features a plant (or plant cell, plant tissue, plant organ, or plant component) including a recombinant transgene capable of expressing a kinase domain of a MAPKKK, wherein the transgene is expressed in the transgenic plant under the control of a promoter that is functional in a plant cell. In

preferred embodiments, the transgene includes a kinase domain which is obtained from a plant. In yet other preferred embodiments, the invention features a kinase domain which is obtained from a fungus (e.g., a yeast) or an animal (e.g., a mammal). In still other preferred embodiments, the transgene consists essentially of the kinase domain.

In related aspects, the invention features seeds and cells from a plant which include a recombinant transgene capable of expressing a kinase domain of a MAPKKK.

In still other related aspects, the invention features a vector (e.g., an expression vector) including a promoter functional in plant cells operably linked to a gene encoding a MAPKKK polypeptide and a cell (e.g., a plant cell or a prokaryotic cell such as *Agrobacterium*) that includes the vector. In preferred embodiments, the gene encodes a polypeptide that consists essentially of a kinase domain of a MAPKKK (e.g., a kinase domain from a plant MAPKKK such as NPK1 or an ANP) or a genetically engineered chimeric polypeptide that includes such a kinase domain.

In general, the kinase domain used in the methods or plants (e.g., transgenic plants or plants that are bred using a transgenic plant) of the invention is generally expressed by itself, as a MAPKKK polypeptide or kinase domain-containing fragment thereof, or as part of a genetically engineered chimeric polypeptide. Useful kinase domains include those that are capable of activating a gene involved in a stress response, repressing early auxin gene expression, or altering seed development. Exemplary kinase domains include, without limitation, those that are substantially identical to the kinase domains of NPK1 or an ANP (e.g., ANP1, ANP2, or ANP3) or AtMEKK1. Preferably, the methods and plants of the invention specifically utilize the kinase domain of NPK1 or ANP1. In other preferred embodiments, a full-length MAPKKK polypeptide or a kinase domain-containing fragment thereof that is substantially identical to any one of NPK1, ANP1, ANP2, or ANP3 is utilized.

The DNA encoding the kinase domain is, in general, constitutively expressed. However, if desired, the kinase domain is inducibly expressed, or such a domain is

expressed in a cell-specific, tissue-specific, or organ-specific manner. Moreover, the kinase domain can also be expressed under cycling conditions (e.g., cell cycle or circadian conditions).

Exemplary plants which are useful in the methods of the invention, as well as for generating the transgenic plants (or plant cells, plant components, plant tissues, or plant organs) of the invention, include dicots and monocots, such as sugar cane, wheat, rice, maize, sugar beet, barley, manioc, crucifer, mustard, potato, soybean, sorghum, cassava, banana, grape, oats, tomato, millet, coconut, orange, rye, cabbage, apple, eggplant, watermelon, canola, cotton, carrot, garlic, onion, pepper, strawberry, yam, papaya, peanut, onion, legume, bean, pea, mango, and sunflower.

By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 40%, preferably 50%, more preferably 80%, and most preferably 90%, or even 95% sequence identity to a reference sequence (for example, the amino acid sequences of the kinase domains or full-length MAPKKK polypeptides of NPK1, ANP1, ANP2, or ANP3 or to their respective nucleic acid sequences (Figs. 11, 12, 13, 14, 15, and 16; SEQ ID NOS: 7-22). For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids or greater. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides or greater.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, FastA, or PILEUP/PRETTYBOX programs). Such software matches

identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "obtained from" is meant isolated from or having the sequence of a naturally-occurring sequence (e.g., a cDNA, genomic DNA, synthetic DNA, or combination thereof).

By "recombinant" is meant a nucleic acid (e.g., DNA) that, is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid of the invention is derived, flank the gene. The term therefore includes, for example, a gene or fragment thereof that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a nucleic acid which is part of a hybrid gene encoding additional polypeptide sequence.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) a MAPKKK kinase domain (e.g., NPK1, ANP1, ANP2, or ANP3).

By "reporter gene" is meant a gene whose expression may be assayed; such genes include, without limitation, β-glucuronidase (GUS), luciferase (LUC), chloramphenicol transacetylase (CAT), green fluorescent protein (GFP), and β-galactosidase.

By "a promoter functional in a plant cell" is meant any minimal sequence sufficient to direct transcription in a plant cell. Included in the invention are promoter

elements that are sufficient to render promoter-dependent gene expression controllable for cell-, tissue-, or organ-specific gene expression, or elements that are inducible by external signals or agents (for example, light-, pathogen-, wound-, stress-, or hormone-inducible elements or chemical inducers) or elements that are capable of cycling gene transcription; such elements may be located in the 5' or 3' regions of the native gene or engineered into a transgene construct.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

By "plant cell" is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. Such a cell also requires a cell wall if further propagation is desired. Plant cell, as used herein, includes, without limitation, algae, cyanobacteria, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell which includes a nucleic acid sequence (e.g., a recombinant DNA sequence) which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants and the DNA (transgene) is inserted by artifice into the nuclear or plastidic genome.

By "increasing stress resistance or tolerance" is meant mediating a level of endurance, adaptability, or durability to a stress (e.g., a man-made stress, such as pollution, or an environmental stress, such as drought, salinity, and oxidative and

temperature stresses) in a transgenic plant which is greater than that exhibited by a control plant (for example, a non-transgenic plant). Preferably, the level of stress resistance or tolerance in a transgenic plant (or transformed plant cell, plant component, plant tissue, or plant organ) of the invention is at least 5%, 10%, or 20% (and preferably 30% or 40%) greater than the tolerance to a stress exhibited in a non-transgenic control plant (or control plant cell, plant component, plant tissue, or plant organ). In other preferred embodiments, the level of stress resistance or tolerance to a stress is 50% greater, 60% greater, and more preferably even 75% or 90% greater than a control plant, with up to 100% above the level of tolerance as compared to a control plant being most preferred. The level of stress resistance or tolerance is measured by conventional methods used to determine plant growth and response to stress. For example, the level of stress tolerance to salinity may be determined by comparing physical features and characteristics (for example, plant height and weight, leaf area, plant water relations, ability to flower, ability to generate seeds, and yield/productivity) of transgenic plants and non-transgenic control plants.

The invention provides a number of important advances and advantages for the protection of plants against environmental stress, such as drought, salt, oxidative damage, and temperature. In addition, the invention provides a means for blocking auxin-inducible gene expression and its concomitant responses affecting plant growth and development. Furthermore, the invention is useful for altering seed development (e.g., for the production of seedless fruits or vegetables), as well as for manipulating endosperm or embryo development. Furthermore, the methods of the invention are advantageous because a kinase domain of MAPKKK is relatively unstable which allows for convenient transgene manipulation, thereby avoiding undesirable side effects

Moreover, the invention facilitates an effective and economical means to improve agronomically important traits of plants for tolerating the effects of dehydration, salinity, cold, and heat. The invention provides for increased production efficiency, as

well as for improvements in quality and yield of crop plants and ornamentals. Thus, the invention contributes to the production of high quality and high yield agricultural products; for example, fruits, ornamentals, vegetables, cereals, and field crops. Genetically-improved seeds and other plant products that are produced using plants expressing the genes and methods described herein also render farming possible in areas previously unsuitable for agricultural production. The invention further provides a means for mediating the expression of stress-related protective proteins (e.g., glutathione S-transferase, asparagine synthetase, or a heat shock protein) that enable a plant to tolerate the effects of environmental stress. For example, transgenic plants constitutively expressing a kinase domain of a MAPKKK are capable of turning on a plant's stress signal transduction pathway by activating the expression of multiple stress-related proteins, which, in turn, enhances the plant's tolerance to multiple stress conditions. Expression of these gene products therefore obviates the need to express individual stress-related genes as a means to promote plant defense mechanisms against adverse environmental conditions.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

The drawings will first be described.

Drawings

Figure 1A is a panel of photomicrographs showing auxin responses in maize protoplasts. The protoplasts were transfected with plasmid DNA carrying either the "GH3-sGFP" or "CAB5-sGFP" auxin-response reporter construct and incubated without or with auxin. Protoplasts expressing GFP were bright green under UV light. Untransfected and uninduced protoplasts showed only blue and pink autofluorescence.

Figure 1B is a histogram showing that the GH3 promoter and the ER7 auxin responsive element are regulated in maize protoplasts. The protoplasts were transfected with plasmid DNA carrying GH3-GUS (designated "GH3"), ER7-GUS (designated "ER7 "), mutated ER7-GUS (designated "mER7"), or a GUS construct under the transcriptional control of the CaMV 35S minimal (-72) promoter (designated "35Smin"). A construct carrying the maize CAB5 promoter (Ulmasov et al., *Science* 276: 1865-1868, 1997) fused to the luciferase gene (designated "CAB-LUC") was used as an internal control in each transfection. The protoplasts were incubated without or with auxin. In each treatment the GUS activity of the cell lysate was divided by the LUC activity, thereby normalizing the data for variation in experimental conditions (that is, number of cells, transformation efficiency, and cell viability). Because of differences in the basal level of expression, GUS/LUC activity of the protoplasts transfected with each construct and incubated without auxin was set to 1. The results shown were the means of triplicate samples ± SD. All experiments were repeated two to three times with similar results.

Figure 2A is a photograph of an autoradiogram showing the expression of different protein kinases in maize protoplasts.

Figure 2B is a photograph of an autoradiogram showing the phosphorylation activity of different protein kinases.

Figure 2C is a photomicrograph showing that constitutively active NPK1 represses the auxin-inducible GH3 promoter. Maize protoplasts were co-transfected with the GH3-sGFP reporter and an effector construct carrying various protein kinases as indicated or vector DNA (control), and incubated with auxin to induce the GH3 promoter.

Figure 2D is a histogram showing that constitutively active NPK1 represses auxin responsive promoters. Maize protoplasts were co-transfected with GH3-GUS (designated "GH3") or ER7-GUS (designated "ER7") reporter and an effector construct carrying the wild-type (designated "NPK1") or mutated (designated "NPK1mut") kinase domain of NPK1, or vector mutated DNA (designated "control"), and incubated with

auxin. A CAB-LUC construct was used as an internal control in each transfection to normalize the GUS activity. The GUS/LUC activity of the control protoplasts induced by auxin was set to 100%. The results shown were the means of triplicate samples ± SD.

Figure 2E is a panel showing a photograph of the expression levels of NPK protein and the null mutation of NPK1 at various times during heat shock (upper panel) and a histogram showing the activation of the GH3 promoter in the presence or absence or auxin (lower panel). The wild-type (NPK1) or mutated (NPK1mut) kinase domain of NPK1 was fused to a DHA tag (Sheen, *Science* 274: 1900-1902, 1996) and inserted into a plant expression vector with a heat shock inducible promoter (designated "HSP") (Sheen et al., *Plant J.* 8: 777-784, 1995). Protoplasts were co-transfected with the GH3-GUS reporter and HSP-NPK1 or HSP-NPK1mut effector. CAB-LUC was used as an internal control in each co-transfection to normalize the GUS activity. The expression of the NPK1 or NPK1mut protein was induced at 40°C for 10, 20, or 60 minutes. The protoplasts from each treatment were divided equally for protein labeling and immunoprecipitation, and for incubation without or with auxin to measure GUS/LUC activity. The GUS/LUC activity of the transfected protoplasts incubated with auxin without heat shock was set to 100%. The results shown were the means of triplicate samples ± SD. All experiments were repeated three times with similar results.

Figure 3A is a schematic diagram showing different NPK1 constructs. The constructs carry the coding region of (1) kinase domain only, (2) NH_2-terminus and kinase domain, (3) kinase domain and COOH-terminus, and (4) full-length NPK1 protein.

Figure 3B is a photograph of an analysis showing the levels of protein expression of the NPK1 constructs (1, 2, 3, and 4) in maize protoplasts.

Figure 3C is a histogram showing the effect of various NPK1s on the GH3 promoter activity. Maize protoplasts were co-transfected with the GH3-GUS reporter construct and one of the NPK1 constructs (1, 2, 3 or 4) shown in Fig. 3A or vector DNA

(control). CAB-LUC was used as an internal control in each transfection to normalize the GUS activity. The GUS/LUC activity of the control protoplasts in the presence of auxin was set to 100%. The results shown were the means of triplicate samples ± SD. All experiments were repeated three times with similar results.

Figure 4A is a panel showing the results of a MAPK in-gel assay (upper panel) and a histogram showing kinase activity (lower panel) of maize protoplasts expressing different MAPKKKs. Protoplasts were transfected with (1) vector DNA for background control; (2) NPK1 kinase domain construct; (3) NPK1 kinase domain mutant construct; (4) full-length NPK1 construct; and (5) CTR1 kinase domain construct. The radioactivity of the 44 kDa putative MAPK band was quantified using a Phosphorimager and normalized to the signal from the background control.

Figure 4B is a photograph of a gel electrophoretic analysis showing the activity of anti-MAPK immunoprecipitated proteins. Protoplasts were transfected with (1) vector DNA for background control; (2) NPK1 kinase domain construct; and (3) NPK1 kinase domain mutant construct.

Figure 4C is a panel of gel electrophoretic analyses showing that MAPK phosphatase (MKP1) inactivates NPK1-induced MAPK. Protoplasts were co-transfected with NPK1 and various protein phosphatase (PP) constructs. The transfected protoplasts were divided to determine protein expression level (upper panel), and to perform the kinase in-gel assay (lower panel).

Figure 4D is a panel of photomicrographs of maize protoplasts showing that MKP1 abolishes the NPK1 repression of the auxin-inducible transcription. Protoplasts were co-transfected with the GH3-sGFP reporter and NPK1, NPK1 + MKP1, NPK1 + PP1, NPK1 + PP2A, or NPK1 + PP2C, and incubated in a medium with auxin. All experiments were repeated two to three times with similar results.

Figure 5A is a histogram showing the H_2O_2, heat shock, and ABA responses in designated *Arabidopsis* protoplasts. Protoplasts were transfected with GST6-LUC (designated "GST6"), HSP18.2-LUC (designated "HSP18.2"), or RD29A-LUC (designated "RD29A") reporter constructs. The transfected protoplasts were divided (10^5 per sample) and incubated at 23°C without (-) or with (+) 200 μM of H_2O_2, 38°C (heat), or 100 μM ABA for 3 hours. The CaMV35S-GUS reporter construct was used as an internal control in each transfection to normalize data for differences in transfection efficiency and cell viability. LUC/GUS was measured as an indicator of the promoter activities. The induction of the HSP18.2 promoter was about 1000 fold, due to extremely low basal expression level. Data are the results of triplicate samples and three independent experiments.

Figure 5B is a histogram showing that H_2O_2 and heat shock suppress the auxin responsive GH3 promoter. *Arabidopsis* protoplasts were transfected with the GH3-GUS reporter construct, divided (10^5 protoplasts per sample), and incubated in the absence (-auxin) or presence of 1 μM NAA (+ auxin) and 200 μM of H_2O_2, or 100 μM ABA at room temperature or at 38°C (heat) for 3 hours. CaMV35S-LUC reporter construct was used as an internal control. GUS/LUC was measured as an indicator of GH3 promoter activity. Data are the results of triplicate samples and three independent experiments. Similar results were obtained when GH3-LUC reporter was used.

Figure 6A is a photograph of an autoradiogram showing the expression of the ANP kinases. *Arabidopsis* protoplasts were transfected with an effector construct expressing one of the HA-tagged protein kinases: kinase domain of ANP1 (designated "ΔANP1"), kinase domain of ANP2 (designated "ΔANP2"), kinase domain of ANP3 (designated "ΔANP3"), kinase domain of ANP1 mutated in the ATP binding site (designated "ΔANP1m"), and full-length ANP1 (ANP1). The transfected protoplasts were incubated in the presence of [^{35}S]-methionine for 4 hours to allow expression and labeling of the effector proteins. The HA-tagged kinases were immunoprecipitated,

separated by SDS-PAGE, and detected.

Figure 6B is a photograph of an autoradiogram showing that ANPs activate two endogenous MAPKs in *Arabidopsis*. *Arabidopsis* protoplasts were transfected with the ANP constructs described in Fig. 6A or with a vector (control) and incubated for 4 hours to allow expression. Activity of endogenous MAPKs in the transfected cells was detected by an in-gel assay using myelin basic protein (MBP) as a substrate.

Figure 6C is a photograph of an autoradiogram showing that ANP1 induced AtMPK3 and AtMPK6 *in vivo*. *Arabidopsis* protoplasts were transfected with constructs expressing one of the HA-tagged *Arabidopsis* MAPKs (designated "AtMPK2 to 7") alone, or co-transfected with another construct expressing HA-tagged ANP1 kinase domain (designated "ΔANP1"). The transfected cells were divided (10^5 each) to detect protein levels (upper panel) or to assay the MAPK activity after immunoprecipitation by using MBP as a substrate (lower panel). Stars indicate non-specific bands seen after immunoprecipitation.

Figure 6D is a photograph of an autoradiogram showing that stresses activate AtMPK3 and ANP1. *Arabidopsis* protoplasts were transfected with AtMPK3 construct alone or co-transfected with full-length ANP1 (designated "AtMPK3+ANP1") or active ANP1 (designated "AtMPK3+ΔANP1"). Cells were incubated for 4 hours to allow protein expression. The protoplasts (10^5 each) were treated with 200 μM of H_2O_2, 38°C (designated "heat"), 4°C (designated "cold"), 1 μM NAA (designated "auxin"), or 100 μM ABA for 15 minutes The AtMPK3 was immunoprecipitated using an anti-HA antibody and assayed for activity by using MBP as a substrate. All data presented in the figure were repeated at least three times with similar results.

Figure 7A is a histogram showing the response of different dicot promoters to the constitutive expression of the ANP1 kinase domain in *Arabidopsis* protoplasts. Protoplasts were co-transfected with either the NR2-LUC (designated "NR2"), AS1-LUC (designated "AS1"), RD29A-LUC (designated "RD29A), HSP-LUC (designated "HSP"),

CAB2-LUC (designated "Cab2"), CHSP-LUC (designated "CHSP"), or GST6-LUC (designated "GST6") reporter gene constructs and an effector construct carrying the wild-type (kANP1) kinase domain, mutated (NPK1mut) kinase domain of NPK1, or the kinase domain of CTR1 (designated "kCTR1"). A 35S NPKmut-GUS construct was used as an internal control in each transfection to normalize the LUC activity. The LUC/GUS activity of the NPK1mut was set to 1. The results shown were the means of triplicate samples ± SD.

Figure 7B is a histogram showing that ANP1 activates stress-inducible promoters. *Arabidopsis* protoplasts were co-transfected with one of the reporter constructs: GST6-LUC (designated "GST6"), HSP18.2-LUC (designated "HSP18.2"), or RD29A-LUC (designated "RD29A") and one of the effector constructs as described in Fig. 6A, kinase domain of CTR1 (desingated "ΔCTR1"), kinase domain of ASK1 (designated "ΔASK1"), full-length CK1-1 (designated "CK1-1"), or a vector ("control"). The CaMV35S-GUS reporter construct was used as an internal control. Transfected cells were incubated for 6 hours before LUC/GUS was measured as an indicator of the promoter activity. Data are the results of triplicate samples and three independent experiments.

Figure 7C is a histogram showing that ANPs repress the auxin response. *Arabidopsis* protoplasts were co-transfected with the *GH3-GUS* reporter construct and one of the effector constructs as described in Fig. 6A, kinase domain of CTR1 (designated "ΔCTR1"), kinase domain of ASK1 (designated "ΔASK1"), full-length CK1-1 (designated "CK1-1"), or a vector (designated "control"). The CaMV35S-LUC reporter construct was used as an internal control. The transfected protoplasts were incubated for 3 hours to allow effector expression before the induction by 1 μM NAA for 3 hours. GUS/LUC was measured as an indicator of the GH3 promoter activity. Data are the results of triplicate samples and three independent experiments.

Figure 8A is a histogram showing the seed germination frequencies of

different transgenic lines of tobacco expressing NPK1. Wild-type (wt) and three independent transgenic lines (2A, 3B, 4A) were examined. The results shown are the means of triplicate samples, 100 seeds each, ± SD.

Figure 8B is a panel of photomicrographs showing the morphological analysis of wild-type and line 4A transgenic seeds. The wild type (upper panel, labeled 1, 2, 3, and 4) and 4A (lower panel, labeled 5, 6, 7, and 8) seeds were soaked for 24 hours in water. The seeds are shown as a population (1,5), typical single seed (2,6), dissected (3,7), and used for the embryo isolation (4,8). The wild type (3), but not the transgenic (7) seeds, showed abundant endosperm, noticeable after the dissection. At least 10 seeds from each population were analyzed in this study.

Figure 8C is a photograph of an RNA blot analysis of the NPK1 transgene expression in wild-type and transgenic tobacco. RNA was isolated from two week-old seedlings. The NPK1 probe hybridized with the transgene RNA only. The endogenous NPK1 mRNA was not detected. Ubiqutin (designated "UBQ") expression was used as a control.

Figure 8D is a photograph of a protein blot analysis of transgene expression. The same amount of proteins (50 mg per lane), extracted from two week-old seedlings, were fractionated in the 12% SDS-PAGE gel and blotted. HA antibody was used to detect HA-tagged transgene proteins. A tobacco transgenic line overexpressing a HA-tagged MEK protein (MEK) was used as a positive control.

Figure 9 is a photograph showing the drought resistance of transgenic tobacco plants expressing the NPK1 transgene. Wild type tobacco seedlings are shown on the left; seedlings of transgenic tobacco, line NPK1-A4, are shown on the right.

Figure 10A is a photograph showing the stress tolerance of transgenic tobacco plants expressing NPK1. Wild type (WT) and transgenic (2A, 3B, 4A) plants were germinated and grown on a 1/4 MS medium for 3 weeks.

Figure 10B is a photograph showing the tolerance of transgenic tobacco plants

expressing NPK1 to freezing temperature. Wild type (WT) and the transgenic (2A, 3B, 4A) plants were grown on plates for 10 days before freezing termperature treatment (-10°C, 3 hours). The photograph was taken 11 days after treatment.

Figure 10C is a photograph showing salt stress tolerance of transgenic tobacco plants expressing NPK1. Wild type (WT) and transgenic plants (2A, 3B, 4A) were germinated on 1/4 MS medium for 6 days, and then transferred to plates containing 300 mM of NaCl for 3 days. The photograph was taken 11 days after the plants were transferred back to the MS plates without NaCl. The graph represents data from five plates (each plate had 10 plants of each genotype).

Figure 10D is a photograph showing the tolerance of transgenic tobacco plants expressing NPK1 to heat shock. Wild type (WT) and transgenic (2A, 3B, 4A) plants were grown on plates for 10 days before heat treatment (48°C, 45 minutes). The photograph was taken 18 days after treatment. The graph represents the data from five plates (each plate had 10 plants of each genotype).

Figure 11 is a diagram showing the alignment of the predicted amino acid sequences of the MAPKKKs: ANP1L, ANP1S, ANP2, ANP3, and NPK1. Kinase domains of these proteins are double-underlined, and are about 268 amino acids in length. Residues that are conserved in three out of the four proteins except (ANP1S) are shown in white letters on a black background. Short conserved stretches (regions A-E) in the four proteins are underlined. Asterisks indicate the consensus sites of phosphorylation by Cdc2 kinase. Only the most carboxy-terminal five amino acid residues of ANP1S that differ from the amino-acid sequence of ANP1L are shown above it (Nishihama et al., *Plant J.* 12:39-48, 1997).

Figure 12 shows the amino acid sequence and corresponding nucleotide sequence of ANP1 (SEQ ID NOS: 7 and 8).

Figure 13 shows the amino acid sequence and corresponding nucleotide sequence of ANP2 (SEQ ID NOS: 11 and 12).

Figure 14 shows the amino acid sequence and corresponding nucleotide sequence of ANP3 (SEQ ID NOS: 15 and 16).

Figure 15 shows the amino acid sequence and corresponding nucleotide sequence of NPK1 (SEQ ID NOS: 19 and 20).

Figure 16 shows the amino acid sequences of the kinase domains of ANP1 (SEQ ID NO: 9), ANP2 (SEQ ID NO: 13), ANP3 (SEQ ID NO: 15), and NPK1 (SEQ ID NO: 21), as well as their corresponding nucleotide sequences (SEQ ID NOS: 10, 14, 16, 22, respectively).

Overview

As is discussed above, the plant hormone auxin is known to activate many early response genes that are likely responsible for diverse aspects of plant growth and development (Davies, P. J., *In*: Plant hormones, ed., Davies, P.J., pp. 1-12, Kluwer, Dordrecht, Netherlands, 1995; Abel et al., *Plant Physiol.* 111: 9-17, 1996; Walden et al., *Trends Plant Sci.* 1: 335-339, 1996). Here we present surprising evidence that a plant MAPK kinase kinase (MPKKK), NPK1 (Banno et al., *Mol. Cell Biol.* 13: 4745-4752, 1993), which possesses similar structure to the mammalian TAK1 (Yamaguchi et al., *Science* 270: 2008-2011, 1995) and fly PK92B (Wassarman et al., *Gene* 169: 283-284, 1996), activates a MAPK cascade that leads to the repression of early auxin response gene transcription. In addition, we show that a mutation in the kinase domain abolished NPK1 activity, and the presence of the COOH-terminal domain diminished the kinase activity. Moreover, the NPK1 effects on the activation of a MAPK and the repression of early auxin response transcription were specifically eliminated by a MAPK phosphatase (Sun et al., *Cell* 75: 487-493, 1993). We also found that transgenic tobacco plants overexpressing constitutively active NPK1 produced seeds defective in embryo and endosperm development. These results indicated that auxin sensitivity could be balanced by antagonistical signaling pathways (Bellincampi et al., *Plant Cell* 8: 477-487, 1996;

Coenen et al., *Trends Plant Sci.* 2: 351-356, 1997) that employ a distinct MAPK cascade in higher plants.

In addition, we provide results showing that constitutively active ANP kinase domains (e.g., ANP1, ANP2, and ANP3) induced the expression of a number of plant stress-inducible gene promoters. Moreover, we provide evidence that transgenic tobacco plants having constitutively active NPK1 produced seedlings that were drought-resistant, as well as resistant to the effects of salt. Such plants were also found to be resistant to other stresses such as heat shock and freezing temperatures.

The examples provided below are for the purpose of illustrating the invention, and should not be construed as limiting.

Auxin Responses in Maize Protoplasts

A transient expression system using freshly isolated maize mesophyll protoplasts has been developed to elucidate the molecular mechanisms of intracellular signal transduction and gene expression in higher plants (Sheen, *Plant Cell* 2: 1027-1038, 1990). This system has been used successfully to study signal transduction pathways stimulated by sugars, light, and the plant hormone abscisic acid (Sheen, *EMBO J.* 12: 3497-3505, 1993; Jang et al., *Plant Cell* 6: 1665-1679, 1994; Sheen, *Science* 274: 1900-1902, 1996; Sheen, *Proc. Natl. Acad. Sci. USA* 95: 975-980, 1998). To determine whether this system is suitable for the investigation of auxin signaling, we have tested the auxin inducibility of a well-characterized early response gene promoter, GH3 (Hagen et al., *Plant Mol. Biol.* 17: 567-579, 1991), in maize mesophyll protoplasts. Maize protoplasts transfected with a construct carrying the coding region of a synthetic green-fluorescent protein (sGFP) (Chiu et al., *Curr. Biol.* 6: 325-330, 1996) driven by the GH3 promoter ("GH3-sGFP") showed bright fluorescence upon induction with different active auxin forms, NAA (Fig. 1A) or IAA (data not shown) at 1mM, a physiologically relevant concentration. In contrast, we observed that auxin did not affect the expression

of a GFP construct ("CAB-sGFP") that was controlled by the maize chlorophyll a/b binding protein gene promoter (CAB5) (Sheen, *Supra* 2: 1027-1038, 1990) (Fig. 1A).

To confirm the auxin inducibility of the GH3 promoter, we also tested the effect of auxin on the promoter fused to another reporter gene encoding the *E. coli* β-glucuronidase (GUS) in transfected maize protoplasts. The results from these experiments showed that GUS activity that was controlled by the GH3 promoter was also induced by auxin (Fig. 1B), although the GUS reporter gene generated higher background than the GFP reporter gene in maize cells.

To support the idea that the early auxin responses are conserved in higher plants, we tested an auxin responsive DNA element, ER7 (Ulmasov et al., *Science* 276: 1865-1868, 1997), which has been found in the majority of early auxin response gene promoters (Abel et al., *Plant Physiol.* 111: 9-17, 1996; Ulmasov et al., *supra*, 1997). A complementary pair of synthetic oligonucleotides containing the ER7 element was fused upstream of the GUS gene driven by a 35S minimal promoter. This ER7-GUS construct showed auxin inducibility in maize protoplasts, whereas the 35S minimal promoter was found not to be induced by auxin (Fig. 1B). Moreover, when the ER7 element was mutated, it lost its auxin inducibility completely (Fig. 1B), as previously shown in transfected carrot protoplasts (Ulmasov et al., *supra*, 1997). These data clearly demonstrated that maize mesophyll protoplasts responded to physiological levels of auxin and that the early auxin responses are likely conserved in monocot and dicot plants.

Constitutively Active NPK1 Represses Auxin-Inducible Promoters

To determine whether NPK1 (Banno et al., *supra*) is involved in auxin signal transduction, we tested the effect of a constitutively active NPK1 on the activity of the GH3 promoter. It has been shown that MAPKKKs consist of a well-conserved kinase domain and putative regulatory domains. Truncated or naturally occurring MAPKKKs carrying only the kinase domain have been shown to have constitutive kinase activity

(Banno, *supra*; Nishihama et al., *Plant J.* 12: 39-48, 1997). The structure of NPK1 is unique as a MAPKKK with the kinase domain located at the NH_2-terminus. A similar structure has also been found in the mammalian TAK1 involved in TGF-β signaling (Yamaguchi et al., *Science* 270: 2008-2011, 1995), and the fly PK92B with an unknown function (Wassarman et al., *Gene* 169: 283-284, 1996). The kinase domain of NPK1 was tagged with two copies of a hemagglutinin (DHA) epitope (Sheen, *supra*, 1996) and cloned into a plant expression vector with a derivative of the CaMV35S promoter (this promoter is not affected by auxin) and the *nos* terminator (Sheen, *supra*, 1993; Sheen, *supra*, 1996; Sheen, *supra*, 1998). The NPK1 construct was co-transfected with the GH3-sGFP or GH3-GUS construct into maize protoplasts. The expression of the NPK1 kinase domain in transfected maize protoplasts was confirmed by ^{35}S-methionine labeling and immunoprecipitation with an anti-HA antibody (Fig. 2A). The kinase activity of the expressed protein was assayed using casein as a universal substrate (Fig. 2B). Surprisingly, the constitutively active NPK1 was found to block auxin activation of the GH3 promoter (Fig. 2C and 2D).

To show that the kinase activity of NPK1 is necessary for this repression, a null mutation (K109M) was created by site-directed mutagenesis to eliminate the ATP binding site conserved among protein kinases (Sheen, *supra*, 1996). This mutation was found not to affect the expression of the NPK1 protein (Fig. 2A), but completely abolished the protein kinase activity (Fig. 2B) and the negative effect of NPK1 on the GH3 promoter in the presence of auxin (Fig. 2C and 2D).

To demonstrate that the inhibitory effect was specific to NPK1, we next tested the effect of another plant MAPKKK, *Arabidopsis* CTR1, that has been shown to act as a negative regulator of ethylene responses (Kieber et al., *Cell* 72: 427-441, 1993). The kinase domain of CTR1 was expressed and displayed protein kinase activity in maize protoplasts (Fig. 2A and 2B), but did not block auxin signaling (Fig. 2C). In addition, because NPK1 is a serine/threonine protein kinase, we expressed other constitutively

active serine/threonine protein kinases that belong to four different classes (Fig. 2A), and tested their effect on the GH3 promoter. Unlike NPK1, none of the tested protein kinases repressed the auxin-regulated gene expression (Fig. 2C) although they all exhibited protein kinase activities in the system (Fig. 2B). Thus, the effect of NPK1 on auxin signaling was not due to non-specific phosphorylation in plant cells.

In addition to the GH3 promoter, we examined the effect of the constitutively active NPK1 on the well-established auxin responsive DNA element, ER7, that has been described by Ulmasov et al. (*supra*, 1997). NPK1 was found to completely suppress the auxin inducibility of the auxin responsive element (Fig. 2D). However, the activities of many auxin-insensitive promoters, including the promoters of CAB, actin, ubiquitin, and CaMV35S genes, were not affected by NPK1 (data not shown). Taken together, these results indicated that NPK1 plays an important and specific role in the negative regulation of the auxin response genes.

It remained possible that NPK1 was a positive regulator in auxin signaling and that the overexpression of NPK1 mimicked the repression of the auxin response genes by very high levels of auxin (Hagen et al., *supra*). To exclude this possibility, we tested the effect of different NPK1 protein levels on the GH3 promoter activity in the absence or presence of auxin. We used a heat shock promoter (Sheen et al., *supra*, 1995) to control the amount of the NPK1 protein produced by varying the time of heat shock. The null mutation of NPK1 served as a control for the effect of the heat shock. As is shown in Fig. 2E, the expression levels of the constitutively active NPK1 and the null mutant correlated well with the duration of heat shock. The activation of the GH3 promoter was not observed at any level of NPK1 in the absence of auxin, ruling out the possibility that NPK1 could be a positive regulator in auxin signaling. In the auxin treated protoplasts, the reverse correlation between the NPK1 protein levels and the GH3 promoter activity supports the idea that NPK1 acts as a negative regulator in auxin signal transduction (Fig. 2E).

Analysis of the Putative Regulatory Domains of NPK1

One distinct feature of NPK1 is the presence of a short NH_2-terminal sequence and a long COOH-terminal region outside the kinase catalytic domain (Banno et al., *supra*). To investigate the function of regions outside the kinase domain in the NPK1 protein, we created several NPK1 deletions (Fig. 3A) and tested their effect on the GH3 promoter activity. Various deletions of the full-length NPK1, as well as the full-length NPK1, showed similar levels of protein expression in transfected maize protoplasts (Fig. 3B). Deletion of the kinase region alone or the kinase domain plus the short NH_2-terminus was found to inhibit the GH3 promoter more strongly than the deletion carrying the kinase domain with the long COOH-terminus or the full-length NPK1 (Fig. 3C).

NPK1 Activates a MAPK

NPK1, as a MAPKKK , is expected to induce a protein phosphorylation cascade resulting in the activation of a MAPK. Although several plant MAPKs have been shown to be induced by stress, hormone, and elicitor signals (Hirt, *Trends Biol. Sci.* 2: 11-15, 1997; Mizoguchi et al., *Trends Biotech.* 15: 15-19, 1997), their activation by a phosphorylation cascade has never been demonstrated in plant cells. To determine whether the expression of the constitutively active NPK1 activates an endogenous MAPK in maize protoplasts, we performed a standard MAPK activity assay (Mizoguchi et al., *Plant J.* 5: 111-122, 1994; Zhang et al., *Plant Cell* 9: 809-824, 1997; Bogre et al., *Plant Cell* 9: 75-83, 1997) with extracts prepared from protoplasts transfected with NPK1 using myelin basic protein (MBP) as a substrate. As shown in Fig. 4A, protoplasts which were transfected with the constitutively active NPK1 had about eight-fold higher 44 kDa kinase activity than protoplasts transfected with the NPK1 null mutation or plasmid DNA carrying no plant genes. This result suggested that the expression of the constitutively active NPK1 resulted in activation of a MAPK. Apparently, a MAPKK was already

present in maize protoplasts and sufficient to relay phosphorylation from MAPKKK (NPK1) to the 44 kDa MAPK. The expression of the full-length NPK1 increased the putative MAPK activity only three fold (Fig. 4A). These results are consistent with the observation that the full-length NPK1 has less effect and the null NPK1 protein has no effect on the repression of the GH3 promoter in the presence of auxin (Fig. 2C, 2D, and 2E; Fig. 3C). As a control, the constitutively active CTR1 also activated an endogenous kinase (Fig. 4A), suggesting the existence of another unrelated MAPK cascade in maize protoplasts. We also observed that the constitutively active CTR1, but not NPK1, could repress ethylene responsive GCC1 enhancer activity more than ten fold in *Arabidopsis* protoplasts, consistent with the proposed role of CTR1 as a negative regulator in the ethylene signaling pathway (Kieber et al., *Cell* 72: 427-441, 1993; Sheen, unpublished).

To verify that NPK1 expression resulted in the activation of a MAPK, we performed kinase activity assays with the proteins immunoprecipitated with an antibody raised against two conserved domains of a mammalian MAPK. The MAPK activity of the protoplasts transfected with the constitutively active NPK1 was significantly higher than that of the cells transfected with the NPK1 null mutant (Fig. 4B). These data are consistent with the results of the MAPK in-gel assay (Fig. 4A), and demonstrate that tobacco NPK1 can induce a kinase cascade in maize protoplasts that activates an endogenous maize MAPK.

To determine whether the 44 kDa MAPK is involved in the repression of early auxin response genes, we tested the effect of a specific MAPK-phosphatase (MKP) that can inactivate MAPKs. Protein phosphatases that can specifically dephosphorylate/inactivate MAPKs have been reported in a variety of eukaryotes and are evolutionarily conserved (Tonks et al., *Cell* 87: 365-368, 1996). A mouse MKP1 (Sun et al., *supra*), highly specific to MAPKs, was cloned into the plant expression vector and expressed in maize protoplasts (Fig. 4C). The expression of MKP1 resulted in the complete elimination of the NPK1 effects, including the NPK1-dependent activation of a

MAPK (Fig. 4C) and the repression of the auxin-inducibility of the GH3 promoter (Fig. 4D). The results suggest that the activation of the 44 kDa MAPK is necessary for the NPK1 dependent repression of transcription. As controls, the expression of other plant protein phosphatases (PP) that belong to the three serine/threonine classes, PP1, PP2A, and PP2C, did not abolish the activation of MAPK by NPK1 (Fig. 4C) or the repression of the GH3 promoter by NPK1 (Fig. 4D), despite the detection of enhanced PP activities in transfected maize protoplasts (Sheen, *supra*, 1993; Sheen, *supra*, 1998) (data not shown). The fact that MKP1 alone does not affect the GH3 promoter (data not shown) supports our current model that a signal(s), antagonizing auxin responses, induces NPK1-like MAPKKKs and leads to the repression of the auxin-inducible transcription.

Stress and Auxin Responses in *Arabidopsis* Protoplasts

To further elucidate the molecular basis of oxidative stress signaling in plants, we have also showed that an *Arabidopsis* protoplast transient expression system is useful to investigate multiple stress responses. Three *Arabidopsis* stress responsive promoters, glutathione S-transferase GST6 (Chen et al., *Plant J.* 10: 995-966, 1996), heat shock HSP18.2 (Takahashi and Komeda, *Mol. Gen. Genet.* 219: 365-372, 1989), and the abscisic acid (ABA) responsive promoter *RD29A* (Yamaguchi-Shinozaki et al., *Plant Physiol.* 101: 1119-1120, 1993; Ishitani et al., *Plant Cell* 9: 1935-1949, 1997), were fused to the luciferase (LUC) reporter and tested for their responses in transfected mesophyll protoplasts. The GST6, HSP18.2, and RD29A promoters were activated by H_2O_2, heat, and ABA, respectively, in protoplasts (Fig. 5A) as demonstrated previously in intact plants (Chen et al., *supra*; Takahashi and Komeda, *supra*; Yamaguchi-Shinozaki et al., *supra*; Ishitani et al., *supra*). Several GST genes, including GST6, have been shown to be induced by high and toxic concentrations of plant growth hormone auxin, as well as by physiologically inactive auxin analogs, heavy metals, and numerous stresses (Chen et al., *supra*; Ulmasov et al., *Plant Mol. Biol.* 26: 1055-1064, 1994; Abel and Theologis, *Plant*

Physiol. 111: 9-17, 1996; Sitbon and Perrot-Rechenmann, *Physiol. Plantarum* 100: 443-455, 1997; Guilfoyle et al., *Plant Physiol.*, 118: 341- 347, 1998, Marrs, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 127- 158, 1996). This non-specific induction of *GSTs* separates them from other auxin responsive genes that are only induced by low physiological levels of active auxin, and indicates that stress rather than auxin is responsible for the activation of the *GST* genes.

H_2O_2 and Heat Shock Suppress the Auxin Responsive GH3 Promoter

H_2O_2, heat, and ABA can arrest cell cycle and plant growth (Inzé and Van Montagu, *supra*; Bolwell and Wojtaszek, *supra*; Lamb and Dixon, *supra*; Noctor and Foyer, *supra*; Leung et al., *supra*; Cheikh and Jones, *Plant Physiol.* 106, 45-51, 1994; Reichheld et al., *Plant J.* 17: 647-656, 1999), the processes promoted by auxin (Key, *BioEssays* 11: 52-58, 1989; Garbers and Simmons, *Trend Cell Biol.* 4: 245-250, 1994; Walker and Estelle, *Curr. Opinion Plant Biol.* 1: 434- 439, 1998; Leyser, *Curr. Biol.* 8: R305-R307, 1998). This suggests a connection between stress and auxin signaling; however, a molecular basis of the crosstalk is unknown. We tested the effects of these stresses on the activity of the auxin responsive promoter, GH3 (Hagen et al., *supra*; Liu et al., *supra*). In *Arabidopsis* protoplasts, physiological concentrations of auxin, 1 μM NAA (Fig. 5B) or 1 μM IAA (data not shown), dramatically increased GH3 promoter activity. The kinetics and magnitude of GH3 promoter activation in *Arabidopsis* protoplasts were comparable to those previously reported in other systems (Hagen et al., *supra*; Liu et al., *supra*). Both H_2O_2 and heat, but not ABA, severely abolished the auxin response (Fig. 5B). The same stress treatments had no significant effects on the CaMV35S promoter activity as an internal control or on ubiquitin promoter UBQ10 activity as a parallel control (data not shown). The repression of the auxin early response gene promoter is therefore likely due to the activation of a specific stress signaling pathway that is common to H_2O_2 and heat, two representative oxidative stress signals

(Inze and Van Montagu, *supra*; Bolwell and Wojtaszek, *supra*; Lamb and Dixon, *supra*; Noctor and Foyer, *supra*). In contrast, the stress hormone ABA did not appear to interfere with auxin signaling in leaf cells.

ANP1 Initiates a Stress MAPK Cascade

In many eukaryotes, the transduction of H_2O_2 and heat stress signals is controlled by protein phosphorylation involving MAPKs (Kyriakis and Avruch, *J. Biol. Chem.* 271: 24313-24316, 1996; Tuomainan et al., *Plant J.* 12: 1151-1162, 1997; Gustin et al., *Microbiol. Mol. Biol. Review* 62: 1264-1300, 1998; Morimoto, *Genes Develpm.* 12: 3788-3796, 1998; Morimoto and Santoro, *Nature BioTech.* 16: 833-838, 1998; Schoffl et al., *Plant Physiol.* 117: 1135-1141, 1998). MAPK and immediate upstream activators, MAPKK and MAPKKK, constitute a functionally interlinked MAPK cascade (Kyriakis and Avruch, *supra*; Tuomainan et al., *supra*; Gustin et al., *supra*). Since the activated tobacco MAPKKK, NPK1 (Banno et al., *supra*), initiated a MAPK cascade that represses auxin early response gene expression (as described herein), we reasoned that this MAPK cascade could also represent a molecular link between oxidative stress and auxin signal transduction. Three *Arabidopsis* NPK1-like MAPKKKs, ANP1-3, share high homology in both their kinase and regulatory domains (Nishihama et al., *Plant J.* 12: 39-48,1997). The regulatory domains of MAPKKKs interact mostly with upstream regulators, whereas the kinase domain forms a complex with the substrate, a specific MAPKK (Xu et al., *Proc. Natl. Acad. Sci. USA* 92: 6808-6812, 1995; Shibuya et al., *Science* 272: 1179-1182, 1996; Clark et al., *Proc. Natl. Acad. Sci. USA* 95: 5401-5406, 1998; Ichimura et al., *Biochem. Biophys. Res. Comm.* 253: 532-543, 1998; Posas and Saito, *EMBO J.* 17: 1385-1394, 1998; Saitoh et al., *EMBO J.* 17: 2596-2606, 1998; Xia et al., *Genes Develop.* 12: 3369-3381, 1998; Yuasa et al., *J. Biol. Chem.* 273: 22681-22692, 1998). Deletions of the regulatory domains, as a result of genetic manipulations, naturally occurred alternative splicing, or proteolytic cleavage, increase MAPKKK activity (Banno et al., *supra*; Xu et

al., *supra*, 1995; Shibuya et al., *supra*, 1996; Clark et al., *supra*; Ichimura et al., *supra*. 253: 532-543, 1998; Posas and Saito, *supra*; Saitoh et al., *supra*; Xia et al., *supra*; Yuasa et al., *supra*; Deak et al., *supra*).

ANPs Activate Two Endogenous MAPKs

We first verified that ANPs could activate endogenous MAPKs in *Arabidopsis*. Coding regions of full length (repressed), kinase domain (constitutively active), or mutated (kinase-inactive) ANPs were fused to the haemagglutinin (HA) epitope tag and expressed in *Arabidopsis* protoplasts (Fig. 6A).

Constitutively active ANPs activated two putative endogenous MAPKs in transfected protoplasts (Fig. 6B). Moreover, a mutation in the ATP binding site abolished, and the presence of the regulatory domains diminished, the ability of ANP1 to activate the putative MAPKs. The sizes of the ANP-activated kinases are similar to those reported for plant MAPKs (Hirt, *Trends Biol. Sci.* 2: 11-15, 1997; Machida et al., *Critic Rev. Plant Sciences* 16: 481-496, 1997; Zhang and Klessig, *Plant Cell* 9: 809-824, 1997; Mizoguchi et al., *Trends BioTech.* 15: 15-19, 1997; Jonak et al., *Cell Mol. Life Sci.* 55: 204-213,1999).

ANPs1 Induce AtMKP3 and AtMPK6 *in vivo*

To identify downstream MAPKs of the ANP-mediated MAPK cascade, constitutively active ANP1 was co-transfected with one of six Arabidopsis MAPKs (AtMPKs), representing three different classes (Hirt, *supra*; Machida et al., *supra*; Zhang and Klessig, *supra*; Mizoguchi et al., *supra*; Jonak et al., *supra*). The active ANP1 initiated a MAPK cascade that could be assayed by measuring the activity of an individual epitope-tagged AtMPK after immunoprecipitation (Fig. 6C). Constitutively active ANP1 slightly changed the mobility of AtMPK3 and AtMPK6 detected by SDS-PAGE, suggesting phosphorylation of these MAPKs (Fig. 6C, upper panel). Notably,

active ANP1 dramatically increased the activity of only these two MAPKs (Fig. 6C, lower panel). Active ANP2 and ANP3, but not another MAPKKK, CTR1 (Kieber et al., *Cell* 72: 427-441, 1993), also induced AtMPK3 and AtMPK6 activity (data not shown), indicating that CTR1 and ANPs activate different MAPK cascades. AtMPK3 and AtMPK6 belong to the class of MAPKs implicated in both stress and pathogen signal transduction in many different plant species (Hirt, *supra*; Machida et al., *supra*; Zhang and Klessig, *supra*; Mizoguchi et al., *supra*; Jonak et al., *supra*). The ability of ANPs to activate stress-related MAPKs indicates that ANP-mediated MAPK cascade is involved in stress signaling.

Stresses Activate AtMKP3 and ANP1

To define the stress signals that can regulate the MAPK cascade, HA epitope-tagged AtMPK3 was transfected into *Arabidopsis* protoplasts, and the protoplasts were then challenged with different stresses. Phosphorylation activity of AtMPK3 was measured after immunoprecipitation with an anti-HA antibody. Several stress signals, including H_2O_2 or heat, but not auxin, activated AtMPK3 (Fig. 6D, left). H_2O_2 or heat also activated AtMPK6 (data not shown). However, when the full-length ANP1 protein was ectopically expressed, only these two stresses, but not other stress stimuli, could further enhance the activation of AtMPK3 (Fig. 6D, center). The fact that H_2O_2 and heat each induced the full-length ANP1 activity to the level of the constitutively active ANP1 (Fig. 6D, right) indicates that ANP1 functions in mediating H_2O_2 and heat stress signal transduction. Induction of AtMPK3 by stimuli unrelated to oxidative stress is probably mediated by an ANP-independent pathway (Fig. 6D, left).

ANP1 Activates Stress-Inducible Promoters

To determine whether a plant MAPKKK, such as ANP1 (Nishihama et al. *Plant J.* 12: 39-48, 1997), is involved in stress signal transduction, we have tested the

effect of a constitutively active ANP1 kinase domain on the activity of several different dicot promoters. This was achieved by introducing into *Arabidopsis* protoplasts a transgene construct consisting of the firefly luciferase coding sequence (LUC) under the control of different dicot promoters. The promoters tested were the nitrate reductase, NR2, promoter from *Arabidopsis* (Lin et al., *Plant Physiol.* 106: 477-484, 1994); the asparagine synthetase, AS1, promoter (Neuhaus et al., *EMBO J.* 16: 2554-2564, 1997); the RD29A *Arabidopsis* stress-responsive promoter (Ishitani et al., *Plant Cell* 9: 1935-1949, 1997); the *Arabidopsis* HSP heat shock promoter (Sheen et al., *Plant Journal* 9: 777-784, 1995; Takahashi et al., *Plant J.* 2: 751-761, 1992); the *Cab2* promoter (Mitra et al. *Plant Mol. Biol.* 12: 169-179, 1989); the chalcone synthase gene promoter (Feinbaum et al., *Mol. Cell Biol.* 8: 1985-1992, 1988); and the H_20_2-inducible glutathione S-transferase promoter (GST) from *Arabidopsis* (Chen et al., *Plant J.* 10: 955-966, 1996). The kinase domain of ANP1 was cloned into a plant expression vector with a derivative of the 35S promoter and the *nos* terminator (Sheen, *Science* 274: 1900-1902, 1996). The ANP1 construct was co-transfected with one of the dicot promoter reporter gene construct and assayed according to standard methods. Surprisingly, the constitutively active ANP1 kinase domain was found to activate the expression of the AS1, HSP, and GST6 promoters (Fig. 7A). Constitutive expression of either the mutated NPK1 kinase domain or the CTR1 kinase domain had no effect on the expression of the dicot reporter genes.

To provide further evidence for the involvement of ANPs in specific stress signaling, we tested the effect of the constitutively active ANP1 on the activity of the GST6, HSP18.2, and RD29A promoters. The active ANP1 could substitute for H_2O_2 and heat to induce the GST6 and HSP18.2 promoters respectively, but did not change the expression of the ABA, cold, or drought responsive RD29A promoter (Fig. 7B). The activation of the GST6 and HSP18.2 promoters required ANP kinase activity since a single amino acid mutation in the ATP binding site completely abolished the ANP1 effect on the promoters. However, the activation was not due to non-specific protein

phosphorylation because three other *Arabidopsis* protein kinases, including another constitutively active MAPKKK, CTR1 (Kieber et al., *supra*), did not affect the promoters' activities. The tested protein kinases were expressed equally well and were at least as active as ANP-like MAPKKKs in transfected cells (as described herein). These results reinforce a role of ANP1 in H_2O_2 and heat signal transduction. However, since ANP1-mediated induction of the HSP18.2 promoter was lower than that obtained by heat shock (Fig. 5A), both ANP-dependent and ANP-independent pathways are probably required to fully activate the heat shock promoter. Since oxidative stress can induce heat shock responsive genes (Morimoto, *supra*; Morimoto and Santoro, *supra*; Schoffl et al., *supra*; Banzet et al., *Plant J.* 13: 519-527, 1998; Storozhenko et al., *Plant Physiol.* 118: 1005-1014, 1998; Zhong et al., *Mol. Cell* 2: 101-108, 1998; Landry and Huot, *Biochem Soc. Symp.* 64: 79-89, 1999), active oxygen species generated by heat shock (Inze and Van Montagu, *supra*; Bolwell and Wojtaszek, *supra*; Lamb and Dixon, *supra*; Noctor and Foyer, *supra*) might be responsible for ANP-dependent activation of the promoter.

ANPs Repress the Auxin Response

To determine whether ANPs can mimic H_2O_2 and heat to repress auxin signaling, we tested the effect of the kinases on GH3 promoter activity. Constitutively active ANP1, ANP2, and ANP3, but not other tested protein kinases, effectively suppressed the GH3 promoter induction by auxin (Fig. 7C). The results suggest that *Arabidopsis* ANPs are orthologs of the tobacco NPK1 that can suppress auxin signaling (as described herein). Thus, similar to H_2O_2 and heat, the constitutively active ANPs can repress the auxin inducible promoter and induce expression of the GST and HSP genes (Figs. 5A, B and Figs. 7B, C).

Analyses of Transgenic Tobacco Plants Expressing NPK1

To assess the function of NPK1 at a whole plant level, we generated

transgenic tobacco plants ectopically overexpressing the constitutively active NPK1. It was anticipated that overexpression of NPK1, as an auxin antagonist, could be lethal. We obtained transgenic plants through three independent transformation experiments. We observed that some seeds from several independent NPK1 transgenic lines never germinated, whereas seeds from the wild type control (Fig. 8A) and many other tobacco lines carrying other transgenes (data not shown) germinated normally. In one line, designated 4A, more than 75% of the seeds did not germinate under any conditions. A closer examination revealed that some transgenic seeds exhibited underdeveloped embryo and endosperm (Fig. 8B). Importantly, the number of defective seeds in each line correlated with the level of transgene expression (Fig. 8D), suggesting that the seed phenotype was due to transgene expression. Although the majority of the transgenic seeds that survived expressed the NPK1 mRNA (Fig. 8C), they produced mostly wild-type looking plants. However, we could not detect the ectopic HA-tagged NPK1 protein in these normal-looking transgenic plants after numerous protein blot analyses, while the control transgenic line expressing the HA-tagged MEK1 showed a strong signal (Fig. 8D). We hypothesize that the truncated NPK1 protein is unstable and cannot accumulate to a level required for causing grossly abnormal growth. This is in agreement with a recent report that a mammalian MAPKKK MEKK1 is degraded rapidly after processing and activation (Widmann et al., *Mol. Cell. Biol.* 18: 2416-2429, 1998). In addition, it was reported that in tobacco cells the NPK1 protein is subjected to a fast turn-over after activation specifically at the end of M phase in the cell cycle (Machida et al., 40th NIBB Conference "Stress responses", 1998), and is detectable at low levels only in fast-growing tissues (Banno et al., *supra*). Thus, accumulation of NPK1 protein might be tightly regulated in plants. This likely explains why the most dramatic effect of NPK1 during embryogenesis and seed development were observed when rapid cell division occurs and more NPK1 proteins may accumulate to block cell cycle progress. The auxin requirement for embryogenesis in plants has been demonstrated (Mordhorst et al.,

Genetics 149: 549-563 1998). Similarly, ectopic activation of a MAPK cascade disrupts *Xenopus* embryo development by inducing mitotic arrest specifically at the M phase (Takenaka et al., *Science* 280: 599-602, 1998).

Transgenic Tobacco Plants Expressing NPK1 Are Resistant to Drought and Excess Salt

Transgenic tobacco plants overexpressing the constitutively active NPK1 were found to be resistant to limited water availability when compared to non-transgenic plants (Fig. 9). In addition, transgenic tobacco seeds constitutively expressing the NPK1 gene were also observed to germinate and grow under high salt conditions (150 mM NaCl), as well as to thrive after exposure to oxidative and heat stresses.

Stress Tolerance of Transgenic Tobacco Plants Ectopically Expressing Active NPK1

GSTs and HSPs encode conjugation enzymes and molecular chaperones, respectively. They play essential roles in detoxification and stabilization of damaged proteins and assisting cell recovery from stresses (Marrs, *supra*; Morimoto, *supra*; Morimoto and Santoro, *supra*; Schoffl et al., *supra*). Constitutive expression of GSTs or HSPs in transgenic tobacco and *Arabidopsis* can make plants more resistant to different stresses, such as salt and heat (Tarczynski et al., *Science* 259: 508-510, 1993; Kishor et al. *Plant Physiol.* 108: 1387-1394, 1995; Lee et al. *Plant J.* 8: 603-612, 1995; Ishizaki-Nishizawa et al., *Nature BioTech.* 14: 1003-1006, 1996; Roxas et al., *Nature BioTech.*15: 988-991, 1997; Prandl et al., *Mol. Gen. Genet.* 258: 269-278, 1998; Jaglo-Ottosen et al., *Science* 280: 104-106, 1998; Liu et al. *Plant Cell* 10: 1391-1406, 1998; Pardo et al., *Proc. Natl. Acad. Sci. USA* 95: 9681-9686, 1998; Pei et al., *Science* 282: 287-290, 1998). Since constitutively active ANP1 induces expression of GST6 and HSP18.2 (Fig. 7B), it is possible that transgenic plants ectopically expressing the active ANP-like protein might be more tolerant to such stresses.

Several transgenic tobacco lines (2A, 3B, 4A), expressing different levels of

the constitutively active tobacco ANP ortholog, NPK1 (as described herein), were examined. Phenotypically, the transgenic plant did not differ from wild type plants under normal growth conditions (Fig. 10A). However, transgenic plants grew more vigorously than did the wild type plants in the presence of 150 mM NaCl. In addition, only 12% of the wild type, but 46%, 68%, and 80% of 2A, 3B, and 4A plants, respectively, survived a three-day exposure to high salt (300 mM NaCl) (Fig. 10C). NPK1 Transgenic plants were also observed to be tolerant to a 3 hour freezing temperature treatment of -10°C (Fig. 10B). We have also tested the sensitivity of NPK1 transgenic plants to heat shock. Exposure to 48°C heat shock killed all the wild type plants, but 24% of 2A, 68% of 3B, and 74% of 4A plants survived (Fig. 10D). The stress tolerance of these NPK1 transgenic plants was proportional to the level of NPK1 transgene expression (as discussed herein). Thus, similar to tobacco and *Arabidopsis* overproducing GSTs and HSPs (Tarczynski et al., *supra*; Kishor et al. *supra*; Lee et al., *supra;* Ishizaki-Nishizawa et al., *supra*; Roxas et al., *supra*; Prandl et al., *supra*; Jaglo-Ottosen et al., *supra*; Liu et al., *supra*; Pardo et al., *supra*; Pei et al., *supra*), the NPK1 transgenic plants were more tolerant to salt and heat than were wild type plants. Although some of the NPK1 transgenic seeds are defective during embryogenesis (as discussed herein) when auxin signaling plays a crucial role (Michalczuk et al., *Phytochem.* 31: 1097-1103, 1992; Ribnicky et al., *Plant Physiol.* 112: 549-558, 1996; Hardtke and Berleth, *EMBO J.* 17: 1405-1411, 1998; Mordhorst et al., *Genetics* 149: 549, 1998; McGovern et al., 9th Arabidopsis Converence, Madison, USA 1998), the absence of obvious growth defects in post-embryonic development of the transgenic plants suggests that the level of NPK1 expression achieved is not deleterious, but rather beneficial in vegetative tissues. The manipulation of this oxidative stress signaling regulator can protect plant cells from diverse environmental stresses, such as heat and high salt. This approach may even be applied for protection from other environmental stresses, such as UV-B, ozone, photooxidation, herbicide, pathogen, drought, and chilling that also involve oxidative

stress damage (Green and Fluhr, *Plant Cell* 7: 203-212, 1995; Prasad, *Plant J.* 10: 1017-1026, 1996; Willekens et al., *EMBO J.* 16: 4806-4816, 1997; Chamnongpol et al., *Proc. Natl. Acad. Sci USA* 95: 5818-5823, 1998; Schraudner et al., *Plant J.* 16: 235-245, 1998; Karpinski et al., *Science* 284: 654-657,1999). Thus, modulation of MAPKKK activity, such as ANP activity, in vegetative tissues provides a novel strategy for cross protection from multiple stresses in agriculturally important plants.

Role of MAPKKKs

Recently, the analysis of auxin resistant mutants in *Arabidopsis* suggested a crucial role of protein degradation in auxin signaling and cell cycle control. For example, several auxin resistant mutants (*axr1*, *tir1*) seemed to be caused by defects in protein degradation processes (Leyser, *Curr. Biol.* 8: R305-R307, 1998). Many auxin-inducible proteins, e.g. SAUR, Aux/IAA, are highly unstable, and some of them function as negative regulators of auxin mediated transcription (Abel et al., *Plant Physiol.* 111: 9-17, 1996; Guilfoyle, *Trends Plant Sci.* 3: 205-207, 1998; Ulmasov et al., *Plant Cell* 9: 1963-1971, 1997). The experiments described herein provide another indication that cell cycle, protein turn-over, and auxin signaling are interconnected.

It has been shown that conserved MAPK cascades mediate numerous vital functions in mammals and yeast, e.g., cell proliferation, cell death, stress responses, through the regulation of gene expression (Herskowitz, *Cell* 80: 187-197, 1995; Kyriakis et al., *J. Biol.Chem.* 271: 24313-24316, 1996). Here, we have presented the first demonstration that, in plant cells, a MAPKKK can activate a MAPK cascade involved in specific regulation of gene expression, and act as a negative regulator in the auxin signal transduction pathway. The recent finding of three NPK1-like protein kinases in *Arabidopsis* (ANPs) (Nishihama et al., *Plant J.* 12: 39-48, 1997) suggests that this distinct MAPKKK is likely conserved in higher plants. In fact, like NPK1, we have found that the kinase domain of ANP1 specifically suppressed the auxin-inducible GH3

promoter in both maize and *Arabidopsis* protoplasts.

Moreover, we have presented evidence indicating that ANP-like MPKKKs mediate oxidative stress signal transduction in plants. For example, oxidative stress signals, H_2O_2 or heat, can activate the MAPKKK. The active ANPs mimic the oxidative stress signals in inducing stress MAPKs and stress response genes, as well as repressing auxin responsive promoter. Therefore, ANP-mediated MAPK cascade links stress and auxin signaling. The activated cascade might help stressed plants to limit auxin-dependent cell division and cell expansion in order to concentrate on survival needs. ANP proteins are found at high levels in meristematic cells and thought to be involved in cell cycle control (Banno et al., *supra*; Nishihama et al., *supra*; Nakashima et al., *Plant Cell Physiol.* 39: 690-700, 1998; Machida et al., 40th NIBB Conference "Stress responses", 1998). Since activation of the stress-induced MAPK cascades usually leads to stress tolerance, a physiological significance of the ANP-related MAPKKKs might be to protect young dividing cells from harsh environmental conditions that plants face during their lifespan. The protection of dividing tissue from stress damage is crucial for survival because continuous organogenesis from the meristems allows reestablishment of plant life.

Materials and Methods

The above-described results were obtained using the following methods.

Reporter Constructs

The 749 bp soybean GH3 promoter (Hagen et al., *Plant Mol. Biol.* 17: 567-579, 1991) was fused to a synthetic gene encoding green-fluorescent protein (sGFP) (Chiu et al., *Curr. Biol.* 6: 325-330, 1996) to visualize the promoter activity. Synthetic ER7 element, TTGTCTCCCAAAGGGAGACAA (SEQ ID NO:1), or mutated ER7, TTGTCTCCCAAAGGGAGAtAA (SEQ ID NO:2) (Ulmasov et al., *Science* 276:

1865-1868, 1997), was inserted in front of the CaMV 35S minimal promoter (-72) (Sheen, *EMBO J.* 12: 3497-3505, 1993). The synthetic promoters were fused to a GUS-nos gene to create ER7-GUS and mER7-GUS reporter constructs. Three clones of each construct were tested for auxin induction and gave identical results.

Arabidopsis MAPKKKs (ANP1, ANP2, ANP3, and CTR1), MAPKs (AtMPK2 to 7), and serine-threonine protein kinases, ASK1 and CK1-1, were obtained by PCR from an *Arabidopsis* cDNA library. The kinase-inactive ANP1 mutant (K98M) was generated by PCR using the following primers: TCTCGCCGTCAtgCAGGTTCTGATTGC (SEQ ID NO:3) and GCAATCAGAACCTGcaTGACGGCGAGAAG (SEQ ID NO:4). The mutation was confirmed by DNA sequencing. All PCR products were tagged with two copies of the hemagglutinin (DHA) epitope, and inserted into a plant expression vector containing the 35SC4PPDK promoter and the *nos* terminator (as described herein). Three to four independent effector clones were tested and gave identical results.

Effector Constructs

NPK1 and CTR1 were obtained by PCR from tobacco cDNA and an *Arabidopsis* cDNA library, respectively. NPK1 deletions were generated by PCR. The null NPK1 mutant (K109M) was generated by PCR using the following primers: TACTCGCTATAAtGGAGGTTTCGAT (SEQ ID NO:5) and CGCAATCGAAACCTCCaTTATAGCGAGTA (SEQ ID NO:6). The mutation was confirmed by DNA sequencing. The PCR products, the coding regions of the constitutively active protein kinases from *Arabidopsis* (CDPK, APK2, ASK2 (Sheen, *Science* 274: 1900-1902, 1996), CK1-1 (Klimczal et al., *Plant Physiol.* 109: 687-696, 1995)), and the coding regions of protein phosphatases (mouse MKP1 (Sun et al., *Cell* 75: 487-493, 1993), maize PP1 (Smith et al., *Plant Physiol.* 97: 677-683, 1991), maize PP2A (Sheen, *EMBO J.* 12: 3497-3505, 1993), and *Arabidopsis* PP2C (Sheen, *Proc.*

Natl. Acad. Sci. USA 95: 975-980, 1998)) were inserted into a plant expression vector containing the 35SC4PPDK promoter, *nos* terminator, and DHA tag (Sheen, *Science* 274: 1900-1902, 1996; Sheen, *Proc. Natl. Acad. Sci. USA* 95: 975-980, 1998). Three to four independent clones were tested in co-transfection experiments with identical results.

Arabidopsis GST6 (Chen et al., *supra*), *HSP18.2* (Takahashi and Komeda, *supra*), and *RD29A* (Yamaguchi-Shinozaki and Shinozaki, *supra*; Ishitani et al., *supra*), as well as soybean *GH3* (Key, *supra*; Garbers and Simmons, *supra*; Walker and Estelle, *supra*; Leyser, *supra*) promoters were fused to the luciferase gene to create GST6-LUC, HSP18.2-LUC, RD29-LUC, and GH3-LUC reporter constructs, respectively.

Protoplast Transient Expression

The preparation, electroporation, and incubation of etiolated maize mesophyll protoplasts were as described previously (Sheen, *Plant Cell* 2: 1027-1038, 1990; Sheen, *EMBO J.* 12: 3497-3505, 1993). In each electroporation, $2x10^5$ protoplasts were transfected with 30 mg of plasmid DNA carrying a reporter construct alone or with 30 mg of plasmid DNA carrying an effector construct or a vector DNA control. The transfected protoplasts were incubated in medium ($5x10^4$/ml) without (- auxin) or with (+ auxin) 1 mM NAA for 14 hours in the dark. GFP fluorescence was observed using UV light as described previously (Sheen et al., *Plant J.* 8: 777-784, 1995). The GUS (Sheen, *Plant Cell* 2: 1027-1038, 1990) and luciferase (Sheen, *Science* 274: 1900-1902, 1996) assays were carried out with cell lysates from 10^4 protoplasts.

Arabidopsis thaliana, ecotype Bensheim, was grown on B5 medium for 4 weeks. The third pair of leaves were cut into 1.0 mm strips and digested overnight in 1% Cellulase R-10, 0.25% Macerozyme R-10, 0.5 M mannitol, 10 mM $CaCl_2$, 20 mM KCl, 10 mM MES, pH 5.7, and 0.1% BSA. Protoplasts were released by gentle shaking, filtered through a 75μm Nylon mesh, collected by centrifugation, and resuspended in W5 solution (Damm et al., *Mol. Gen. Genet.* 217:6, 1989; Abel and Theologis, *supra*).

Before transfection, protoplasts were resuspended in 0.4 M Mannitol, 15 mM $MgCl_2$ and 4 mM MES, pH 5.7, to a density of 10^6 protoplasts/ml. Typically 0.2 ml of the protoplast suspension was mixed with 30 to 50 µg of plasmid DNA containing reporter and effector constructs and equal volume of 40% PEG solution (Damm et al., *Mol. Gen. Genet.* 217:6, 1989; Abel and Theologis, *supra*). The transfected protoplasts were diluted with W5 solution, collected by centrifugation, and resuspended in the incubation solution (0.5 M Mannitol, 20 mM KCl, 4 mM MES, pH 5.7).

Determination of Effector Expression

Transfected maize protoplasts (10^5) were incubated for 5 hours with [^{35}S]-methionine (200 mCi/ml) before harvesting. The NPK1 protein was less stable than other expressed proteins after long incubation (data not shown). Immunoprecipitation with an anti-HA antibody was performed as described previously (Sheen, *Science* 274: 1900-1902, 1996). The proteins were separated by SDS-PAGE (10%) and visualized by fluorography.

In-Gel Kinase Activity Assay

The transfected protoplasts (10^5) were incubated for 5 hours before harvesting. The kinase in-gel assay was performed as described previously (Zhang et al., *Plant Cell* 9: 809-824, 1997).

Immunoprecipitation Kinase Activity Assay

Cell lysates from 10^5 transfected protoplasts were used for immunoprecipitation with an anti-ERK (PAC) antibody (Transduction Laboratory) (Sheen, *Science* 274: 1900-1902, 1996). The immunoprecipitated proteins were assayed for MAPK activity using MBP as substrate (Bogre et al., *Plant Cell* 9: 75-83, 1997). The [^{32}P]-MBP was separated by SDS-PAGE (15%) and visualized by autoradiography.

Protein Kinase and Phosphatase Activity Assays

Cell lysates from 10^5 transfected protoplasts were used for immunoprecipitation with an anti-HA antibody (Sheen, *Science* 274: 1900-1902, 1996) to bring down the HA-tagged protein kinases. The immunoprecipitated proteins were assayed using casein as substrate. The [^{32}P]-casein was separated by SDS-PAGE (10%) and visualized by autoradiography. PP1, PP2A, and PP2C activity assays using transfected maize cell extracts were described previously (Sheen, *EMBO J.* 12: 3497-3505, 1993; Sheen, *Proc. Natl. Acad. Sci. USA* 95: 975-980, 1998).

Transgenic Plants

A construct including the 35SC4PPDK promoter (Sheen, *EMBO J.* 12: 3497-3505, 1993; Sheen, *Science* 274: 1900-1902, 1996; Sheen, *Proc. Natl. Acad. Sci. USA* 95: 975-980, 1998), kinase domain of NPK1, DHA tag, and *nos* terminator was inserted into pART27 binary vector (Gleave, *Plant Mol. Biol.* 20: 1203-1207, 1992). The resulting plasmid was introduced into *Agrobacterium tumefaciens* EHA105, and the transformation was performed with *Nicotiana tabacum* SR1 leaf discs (Chiu et al., *Curr. Biol.* 6: 325-330, 1996). Several kanamycin-resistant plants were selected from three independent transformation experiments. The kanamycin resistance of T1 progeny plants revealed that the three analyzed independent parental transformants contained more than one copy of the transgene. The seeds were examined under a light microscope. RNA blot and protein blot analyses were performed as described previously (Jang et al., *Plant Cell* 9: 5-19, 1997).

Isolation of Sequences Encoding MAPKKK Kinase Domains

The isolation of additional MAPKKK coding sequences, as well as MAPKKK kinase domains, having the ability to regulate auxin responses (or activate stress responses, or alter seed development) in plants is accomplished using standard strategies

and techniques that are well known in the art.

In one particular example, the tobacco NPK1 sequences (or *Arabidopsis* ANP1, ANP2, or ANP3 sequences) described herein may be used, together with conventional screening methods of nucleic acid hybridization screening, to isolate additional sequences encoding MAPKKK polypeptides (or kinase domain-containing fragements thereof), as well as kinase domains of MAPKKK (Figs. 11, 12, 13, 14, 15, and 16; SEQ ID NOS: 7-22). Such hybridization techniques and screening procedures are well known to those skilled in the art and are described, for example, in Benton and Davis, *Science* 196: 180, 1977; Grunstein and Hogness, *Proc. Natl. Acad. Sci., USA* 72: 3961, 1975; Ausubel et al. *Current Protocols in Molecular Biology*, Wiley Interscience, New York; Berger and Kimmel, *Guide to Molecular Cloning Techniques*, 1987, Academic Press, New York.; and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. In one particular example, all or part of the NPK1 gene (described herein) may be used as a probe to screen a recombinant plant DNA library for genes having sequence identity or similarity to the NPK1 gene or its kinase domain (Figs. 11, 15, and 16). Hybridizing sequences are detected by plaque or colony hybridization according to the methods described below.

Alternatively, using all or a portion of the amino acid sequence of the kinase domain, one may readily design kinase domain-specific oligonucleotide probes, including kinase domain degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either DNA strand and any appropriate portion of the kinase domain sequence. General methods for designing and preparing such probes are provided, for example, in Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience, New York; and Berger and Kimmel, *Guide to Molecular Cloning Techniques*, 1987, Academic Press, New York. These oligonucleotides are useful for kinase domain sequence isolation, either through their use as probes capable of hybridizing to kinase

complementary sequences or as primers for various amplification techniques, for example, polymerase chain reaction (PCR) cloning strategies. If desired, a combination of different oligonucleotide probes may be used for the screening of a recombinant DNA library. The oligonucleotides may be detectably-labeled using methods known in the art and used to probe filter replicas from a recombinant DNA library. Recombinant DNA libraries are prepared according to methods well known in the art, for example, as described in Ausubel et al. (*supra*), or they may be obtained from commercial sources.

As discussed above, kinase domain-specific oligonucleotides may also be used as primers in amplification cloning strategies, for example, using PCR. PCR methods are well known in the art and are described, for example, in *PCR Technology*, Erlich, ed., Stockton Press, London, 1989; *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, Inc., New York, 1990; and Ausubel et al. (*supra*). Primers are optionally designed to allow cloning of the amplified product into a suitable vector, for example, by including appropriate restriction sites at the 5' and 3' ends of the amplified fragment (as described herein). If desired, kinase domain sequences may be isolated using the PCR "RACE" technique, or Rapid Amplification of cDNA Ends (see, e.g., Innis et al. (*supra*)). By this method, oligonucleotide primers based on an kinase domain sequence are oriented in the 3' and 5' directions and are used to generate overlapping PCR fragments. These overlapping 3'- and 5'-end RACE products are combined to produce an intact full-length cDNA. This method is described in Innis et al. (*supra*); and Frohman et al., *Proc. Natl. Acad. Sci. USA* 85: 8998, 1988.

Confirmation of a sequence's relatedness to the kinase domains of the NPK and ANP MAPKKKs may be accomplished by a variety of conventional methods including, but not limited to, sequence comparison of the gene and its expressed product. In addition, the activity of the gene product may be evaluated according to any of the techniques described.

Once a MAPKKK gene or its kinase domain is identified, it is cloned

according to standard methods and used for the construction of plant expression vectors as described below.

Expression Constructs

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. A MAPKKK polypeptide or its kinase domain may be produced in a prokaryotic host, for example, *E. coli*, or in a eukaryotic host, for example, *Saccharomyces cerevisiae*, mammalian cells (for example, COS 1 or NIH 3T3 cells), or any of a number of plant hosts including, without limitation, algae, tree species, ornamental species, temperate fruit species, tropical fruit species, vegetable species, legume species, crucifer species, monocots, dicots, or in any plant of commercial or agricultural significance. Particular examples of suitable plant hosts include, but are not limited to, Conifers, Petunia, Tomato, Potato, Tobacco, *Arabidopsis*, Lettuce, Sunflower, Oilseed rape, Flax, Cotton, Sugarbeet, Celery, Soybean, Alfalfa, *Medicago*, Lotus, *Vigna*, Cucumber, Carrot, Eggplant, Cauliflower, Horseradish, Morning Glory, Poplar, Walnut, Apple, Grape, Asparagus, Rice, Maize, Millet, Onion, Barley, Orchard grass, Oat, Rye, and Wheat. In addition, as is discussed below, expression constructs may be expressed in a transgenic plant to turn on the stress signal transduction pathway to enhance plant tolerance to multiple stress conditions.

Materials for expressing these genes are available from a wide range of sources including the American Type Culture Collection (Rockland, MD); or from any of a number seed companies, for example, W. Atlee Burpee Seed Co. (Warminster, PA), Park Seed Co. (Greenwood, SC), Johnny Seed Co. (Albion, ME), or Northrup King Seeds (Harstville, SC). Descriptions and sources of useful host cells are also found in Vasil I.K., Cell Culture and Somatic Cell Genetics of Plants, Vol I, II, III Laboratory Procedures and Their Applications Academic Press, New York, 1984; Dixon, R.A., Plant

Cell Culture-A Practical Approach, IRL Press, Oxford University, 1985; Green et al., Plant Tissue and Cell Culture, Academic Press, New York, 1987; and Gasser and Fraley, Science 244: 1293, 1989.

The method of transformation or transfection and the choice of vehicle for expression of the MAPKKK polypeptide or its kinase domain will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*supra*); Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989; Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers, 1990; Kindle, K., *Proc. Natl. Acad. Sci., U.S.A* 87: 1228, 1990; Potrykus, I., *Annu. Rev. Plant Physiol. Plant Mol. Biology* 42: 205, 1991; and BioRad (Hercules, CA) Technical Bulletin #1687 (Biolistic Particle Delivery Systems). Expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P.H. Pouwels et al., 1985, Supp. 1987); Gasser and Fraley (supra); Clontech Molecular Biology Catalog (Catalog 1992/93 Tools for the Molecular Biologist, Palo Alto, CA); and the references cited above. Other expression constructs are described by Fraley et al. (U.S. Pat. No. 5,352,605).

Most preferably, a MAPKKK polypeptide or its kinase domain is produced by a stably-transfected plant cell line, a transiently-transfected plant cell line, or by a transgenic plant. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants are available to the public; such vectors are described in Pouwels et al. (*supra*), Weissbach and Weissbach (*supra*), and Gelvin et al. (*supra*). Methods for constructing such cell lines are described in, e.g., Weissbach and Weissbach (*supra*), and Gelvin et al. (*supra*). Typically, plant expression vectors include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (for example, one conferring inducible or constitutive, pathogen- or wound-induced, environmentally- or developmentally-

regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Once the desired nucleic acid sequence encoding a MAPKKK polypeptide or its kinase domain is obtained as described above, it may be manipulated in a variety of ways known in the art. For example, where the sequence involves non-coding flanking regions, the flanking regions may be subjected to mutagenesis.

The kinase domain sequence (or a MAPKKK polypeptide or kinase domain-containing fragment thereof), if desired, may be combined with other DNA sequences in a variety of ways. Such a sequence may be employed with all or part of the gene sequences normally associated with itself. In its component parts, a DNA sequence encoding a MAPKKK polypeptide or its kinase domain is combined in a DNA construct having a transcription initiation control region capable of promoting transcription and translation in a host cell.

In general, the constructs will involve regulatory regions functional in plants which provide for modified production of the regulator protein as discussed herein. The open reading frame coding for the regulator protein or functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory region such as the sequence naturally found in the 5' upstream region of the MAPKKK polypeptide or its kinase domain. Numerous other transcription initiation regions are available which provide for constitutive or inducible regulation.

For applications where developmental, cell, tissue, hormonal, or environmental expression is desired, appropriate 5' upstream non-coding regions are obtained from other genes, for example, from genes regulated during meristem development, seed development, embryo development, or leaf development.

Regulatory transcript termination regions may also be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by

the DNA sequence encoding the MAPKKK polypeptide or any convenient transcription termination region derived from a different gene source. The transcript termination region will contain preferably at least 1-3 kb of sequence 3' to the structural gene from which the termination region is derived. Plant expression constructs having, for example, a MAPKKK protein kinase domain (e.g., the NPK1 kinase domain) as the DNA sequence of interest for expression may be employed with a wide variety of plant life. Such genetically-engineered plants are useful for a variety of industrial and agricultural applications as discussed herein. Importantly, this invention is applicable to dicotyledons and monocotyledons, and will be readily applicable to any new or improved transformation or regeneration method.

An example of a useful plant promoter according to the invention is a caulimovirus promoter, for example, a cauliflower mosaic virus (CaMV) promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, e.g., Odell et al., Nature 313: 810, 1985). The CaMV promoter is also highly active in monocots (see, e.g., Dekeyser et al., Plant Cell 2: 591, 1990; Terada and Shimamoto, *Mol. Gen. Genet.* 220: 389, 1990). Moreover, activity of this promoter can be further increased (i.e., between 2-10 fold) by duplication of the CaMV 35S promoter (see e.g., Kay et al., *Science* 236: 1299, 1987; Ow et al., *Proc. Natl. Acad. Sci., U.S.A.* 84: 4870, 1987; and Fang et al., *Plant Cell* 1: 141, 1989). In addition, the a minimal 35S promoter may also be used as is described herein.

Other useful plant promoters include, without limitation, the nopaline synthase promoter (An et al., *Plant Physiol.* 88: 547, 1988) and the octopine synthase promoter (Fromm et al., *Plant Cell* 1: 977, 1989).

For certain applications, it may be desirable to produce the MAPKKK polypeptide or its kinase domain in an appropriate tissue, at an appropriate level, or at an

appropriate developmental time. For this purpose, there are an assortment of gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, shown to be regulated in response to the environment, hormones, and/or developmental cues. These include gene promoters that are responsible for heat-regulated gene expression (see, e.g., Callis et al., *Plant Physiol.* 88: 965, 1988; Takahashi and Komeda, *Mol. Gen. Genet.* 219: 365, 1989; and Takahashi et al., *Plant J.* 2: 751, 1992), light-regulated gene expression (e.g., the *Arabidopisis Cab2* photosynthetic, leaf specific promoter described by Mitra at el., *Plant Mol. Biol.* 12: 169-179, 1989; the pea *rbcS-3A* described by Kuhlemeier et al., *Plant Cell* 1: 471, 1989; the maize *rbcS* promoter described by Schäffner and Sheen, *Plant Cell* 3: 997, 1991; or the cholorphyll a/b-binding protein gene found in pea described by Simpson et al., *EMBO J.* 4: 2723, 1985), hormone-regulated gene expression (for example, the abscisic acid (ABA) responsive sequences from the *Em* gene of wheat described by Marcotte et al., *Plant Cell* 1: 969, 1989; the ABA-inducible HVA1 and HVA22, and rd29A promoters described for barley and *Arabidopsis* by Straub et al., *Plant Cell* 6: 617, 1994, Shen et al., Plant Cell 7: 295, 1995; and wound-induced gene expression (for example, of *wunI* described by Siebertz et al., *Plant Cell* 1: 961, 1989), organ-specific gene expression (for example, of the tuber-specific storage protein gene described by Roshal et al., *EMBO J.* 6: 1155, 1987; the 23-kDa zein gene from maize described by Schernthaner et al., *EMBO J.* 7: 1249, 1988; or the French bean ß-phaseolin gene described by Bustos et al., *Plant Cell* 1: 839, 1989; the vegetative storage protein promoter (soybean vspB) described by Sadka et al (*Plant Cell* 6: 737-749, 1994)), cycling promoters (e.g., the *Arabidopsis* cdc2a promoter described by Hemerly et al., *Proc Natl Acad Sci USA* 89: 3295-3299, 1992), senescence-specific promoters (e.g., the *Arabidopsis* SAG12 promoter described by Gan et al, *Science*: 270, 1986-1988, 1995), seed-specific promoters (for example, endosperm-specific or embryo-specific promoters), or pathogen-inducible promoters (for example, PR-1 or β-1,3 glucanase promoters).

Plant expression vectors may also optionally include RNA processing signals, e.g, introns, which have been shown to be important for efficient RNA synthesis and accumulation (Callis et al., *Genes and Dev.* 1: 1183, 1987). The location of the RNA splice sequences can dramatically influence the level of transgene expression in plants. In view of this fact, an intron may be positioned upstream or downstream of a MAPKKK polypeptide or its kinase-domain encoding sequence in the transgene to modulate levels of gene expression.

In addition to the aforementioned 5' regulatory control sequences, the expression vectors may also include regulatory control regions which are generally present in the 3' regions of plant genes (Thornburg et al., *Proc. Natl. Acad. Sci. U.S.A.* 84: 744, 1987; An et al., *Plant Cell* 1: 115, 1989). For example, the 3' terminator region may be included in the expression vector to increase stability of the mRNA. One such terminator region may be derived from the PI-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or nopaline synthase signals.

The plant expression vector also typically contains a dominant selectable marker gene used to identify those cells that have become transformed. Useful selectable genes for plant systems include genes encoding antibiotic resistance genes, for example, those encoding resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin, or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-deficient strains. Finally, genes encoding herbicide resistance may be used as selectable markers; useful herbicide resistance genes include the *bar* gene encoding the enzyme phosphinothricin acetyltransferase and conferring resistance to the broad spectrum herbicide Basta® (Hoechst AG, Frankfurt, Germany).

Efficient use of selectable markers is facilitated by a determination of the susceptibility of a plant cell to a particular selectable agent and a determination of the concentration of this agent which effectively kills most, if not all, of the transformed

cells. Some useful concentrations of antibiotics for tobacco transformation include, e.g., 75-100 μg/mL (kanamycin), 20-50 μg/mL (hygromycin), or 5-10 μg/mL (bleomycin). A useful strategy for selection of transformants for herbicide resistance is described, e.g., by Vasil et al., *supra*.

It should be readily apparent to one skilled in the art of molecular biology, especially in the field of plant molecular biology, that the level of gene expression is dependent, not only on the combination of promoters, RNA processing signals, and terminator elements, but also on how these elements are used to increase the levels of selectable marker gene expression.

Plant Transformation

Upon construction of the plant expression vector, several standard methods are available for introduction of the vector into a plant host, thereby generating a transgenic plant. These methods include (1) Agrobacterium-mediated transformation (*A. tumefaciens* or *A. rhizogenes*) (see, e.g., Lichtenstein and Fuller, *In:* Genetic Engineering, vol 6, PWJ Rigby, ed, London, Academic Press, 1987; and Lichtenstein, C.P., and Draper, J,. In: *DNA Cloning*, Vol II, D.M. Glover, ed, Oxford, IRI Press, 1985)), (2) the particle delivery system (see, e.g., Gordon-Kamm et al., *Plant Cell* 2: 603,1990); or BioRad Technical Bulletin 1687, *supra*), (3) microinjection protocols (see, e.g., Green et al., *supra*), (4) polyethylene glycol (PEG) procedures (see, e.g., Draper et al., *Plant Cell* Physiol. 23: 451, 1982; or e.g., Zhang and Wu, *Theor. Appl. Genet.* 76: 835, 1988), (5) liposome-mediated DNA uptake (see, e.g., Freeman et al., *Plant Cell Physiol.* 25: 1353, 1984), (6) electroporation protocols (see, e.g., Gelvin et al., *supra*; Dekeyser et al., *supra*; Fromm et al., *Nature* 319: 791, 1986; *Sheen*, Plant Cell 2: 1027, 1990; or Jang and Sheen, *Plant Cell* 6: 1665, 1994), and (7) the vortexing method (see, e.g., Kindle *supra*). The method of transformation is not critical to the invention. Any method which provides for efficient transformation may be employed. As newer

methods are available to transform crops or other host cells, they may be directly applied.

The following is an example outlining one particular technique, an *Agrobacterium*-mediated plant transformation. By this technique, the general process for manipulating genes to be transferred into the genome of plant cells is carried out in two phases. First, cloning and DNA modification steps are carried out in *E. coli*, and the plasmid containing the gene construct of interest is transferred by conjugation or electroporation into *Agrobacterium*. Second, the resulting *Agrobacterium* strain is used to transform plant cells. Thus, for the generalized plant expression vector, the plasmid contains an origin of replication that allows it to replicate in *Agrobacterium* and a high copy number origin of replication functional in *E. coli*. This permits facile production and testing of transgenes in *E. coli* prior to transfer to *Agrobacterium* for subsequent introduction into plants. Resistance genes can be carried on the vector, one for selection in bacteria, for example, streptomycin, and another that will function in plants, for example, a gene encoding kanamycin resistance or herbicide resistance. Also present on the vector are restriction endonuclease sites for the addition of one or more transgenes and directional T-DNA border sequences which, when recognized by the transfer functions of *Agrobacterium*, delimit the DNA region that will be transferred to the plant.

In another example, plant cells may be transformed by shooting into the cell tungsten microprojectiles on which cloned DNA is precipitated. In the Biolistic Apparatus (Bio-Rad) used for the shooting, a gunpowder charge (22 caliber Power Piston Tool Charge) or an air-driven blast drives a plastic macroprojectile through a gun barrel. An aliquot of a suspension of tungsten particles on which DNA has been precipitated is placed on the front of the plastic macroprojectile. The latter is fired at an acrylic stopping plate that has a hole through it that is too small for the macroprojectile to pass through. As a result, the plastic macroprojectile smashes against the stopping plate, and the tungsten microprojectiles continue toward their target through the hole in the plate. For the instant invention the target can be any plant cell, tissue, seed, or embryo. The DNA

introduced into the cell on the microprojectiles becomes integrated into either the nucleus or the chloroplast.

In general, transfer and expression of transgenes in plant cells are now routine practices to those skilled in the art, and have become major tools to carry out gene expression studies in plants and to produce improved plant varieties of agricultural or commercial interest.

Transgenic Plant Regeneration

Plant cells transformed with a plant expression vector can be regenerated, for example, from single cells, callus tissue, or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues, and organs from almost any plant can be successfully cultured to regenerate an entire plant; such techniques are described, e.g., in Vasil *supra*; Green et al., *supra*; Weissbach and Weissbach, *supra*; and Gelvin et al., *supra*.

In one particular example, a cloned kinase domain of a MAPKKK (or a MAPKKK polypeptide or a kinase-containing fragment thereof) construct under the control of the *nos* promoter and the nopaline synthase terminator and carrying a selectable marker (for example, kanamycin resistance) is transformed into *Agrobacterium*. Transformation of leaf discs (for example, of tobacco or potato leaf discs), with vector-containing *Agrobacterium* is carried out as described by Horsch et al. (*Science* 227: 1229, 1985). Putative transformants are selected after a few weeks (for example, 3 to 5 weeks) on plant tissue culture media containing kanamycin (e.g. 100 µg/mL). Kanamycin-resistant shoots are then placed on plant tissue culture media without hormones for root initiation. Kanamycin-resistant plants are then selected for greenhouse growth. If desired, seeds from self-fertilized transgenic plants can then be sowed in a soil-less medium and grown in a greenhouse. Kanamycin-resistant progeny are selected by sowing surfaced sterilized seeds on hormone-free kanamycin-containing

media. Analysis for the integration of the transgene is accomplished by standard techniques (see, for example, Ausubel et al. *supra*; Gelvin et al. *supra*).

Transgenic plants expressing the selectable marker are then screened for transmission of the transgene DNA by standard immunoblot and DNA detection techniques. Each positive transgenic plant and its transgenic progeny are unique in comparison to other transgenic plants established with the same transgene. Integration of the transgene DNA into the plant genomic DNA is in most cases random, and the site of integration can profoundly affect the levels and the tissue and developmental patterns of transgene expression. Consequently, a number of transgenic lines are usually screened for each transgene to identify and select plants with the most appropriate expression profiles.

Transgenic lines are evaluated for levels of transgene expression. Expression at the RNA level is determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis are employed and include PCR amplification assays using oligonucleotide primers designed to amplify only transgene RNA templates and solution hybridization assays using transgene-specific probes (see, e.g., Ausubel et al., *supra*). The RNA-positive plants are then analyzed for protein expression by Western immunoblot analysis using specific antibodies (see, e.g., Ausubel et al., *supra*). In addition, *in situ* hybridization and immunocytochemistry according to standard protocols can be done using transgene-specific nucleotide probes and antibodies, respectively, to localize sites of expression within transgenic tissue.

In addition, if desired, once the recombinant MAPKKK polypeptide or its kinase domain is expressed in any cell or in a transgenic plant (for example, as described above), it may be isolated, e.g., using affinity chromatography. In one example, an anti-MAPKKK polypeptide antibody (e.g., produced as described in Ausubel et al., *supra*, or by any standard technique) may be attached to a column and used to isolate the polypeptide. Lysis and fractionation of MAPKKK-producing cells prior to affinity

chromatography may be performed by standard methods (see, e.g., Ausubel et al., *supra*). Once isolated, the recombinant protein can, if desired, be further purified, for example, by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, 1980).

Engineering Stress-Protected Transgenic Plants

As discussed above, because constitutive MAPKKK activity has been found to activate stress-inducible gene promoters such as GST6 (Chen et al., *Plant J.* 10: 955-966, 1996), HSP 18.2 (Sheen et al., *Plant Journal* 9: 777-784, 1995; Takahashi et al., *Plant J.* 2: 751-761, 1992), and AS1 (Neuhaus et al., *EMBO J.* 16: 2554-2564, 1997), constructs designed for the expression of a kinase domain of a MAPKKK are useful for generating transgenic plants having an increased level of tolerance to environmental stress. To achieve such tolerance, it is important to express a kinase domain at an effective level. For example, the *Cab* and *RbcS* gene promoters are especially useful for the expression of a kinase domain in leaves; and the 35S CaMV(-90) promoter is useful for the expression of the kinase domain in the roots of a plant. Evaluation of the level of stress protection conferred to a plant by expression of a DNA sequence expressing a kinase domain of a MAPKKK polypeptide is determined according to conventional methods and assays, for example, as described below.

Salt or Osmotic Stresses

In one working example, tissue-specific expression of a kinase domain of a MAPKKK, for example, the NPK1 kinase domain gene, is used in tomato to enhance salt stress tolerance. For example, a plant expression vector is constructed that contains an NPK1 protein kinase domain sequence expressed under the control of a root specific promoter (for example, the 35S CaMV minimal promoter). This expression vector is then used to transform tomato according to standard methods (for example, those described herein). To assess salt tolerance, transformed tomato plants and appropriate

controls are evaluated according to methods described in Lilus et al. (*BioTechnology* 14: 177, 1996) and Tarczynski et al. (*Science* 259: 508, 1993). Transgenic seeds containing the gene are germinated in the presence of various salt or osmotically active solutions to determine whether transgenic seeds demonstrate increased tolerance or resistance to salt stress. Seedlings can also be grown in hydroponic systems and challenged with salt or agents of differing osmotic potentials at different, or all, developmental stages in order to assess the response of a MAPKKK kinase domain-expressing plants to these stresses. Growth and physiological measurements are used to document the differences. Transformed tomato plants having an increased level of salt tolerance relative to control plants are taken as being useful in the invention.

Drought

Transgenic plants expressing a recombinant MAPKKK kinase domain are also assayed for tolerance to drought. Such analyses are preferably done in artificial environments to simulate drought or limited water conditions. In addition, transgenic seeds may be planted outside during times when the natural environment would impose the stress.

Cold

To demonstrate whether kinase domain expression can confer increased germination ability under cool conditions, transgenic seeds expressing a recombinant kinase domain of a MAPKKK polypeptide are germinated under conditions similar to the standard cold germination tests used in the seed industry. Alternatively, transgenic seeds expressing such a kinase domain are planted under cool seed bed conditions made cool by artificial environments or naturally cool seed beds in the field. Additionally, plants expressing the kinase domain are challenged during the seed development period for cool night time temperatures to demonstrate decreased inhibition of leaf or canopy activity as a result of cold stress during this time of crop development. Young transgenic seedlings are grown at low temperature, such as about 15°C, during the light and dark period. The

expression of a recombinant kinase domain in these seedlings not only allows for increased growth, but also allows the seedlings to become photosynthetic under such conditions, as well as to survive and grow.

Frost or Freeze

Transgenic plants expressing a recombinant MAPKKK kinase domain are also assayed for increased freezing tolerance at the seedling stage as well as late season periods. These assays are preferably done in artificial environments to simulate frost or freeze events. In addition, transgenic seeds may be planted outside during times when the natural environment would impose the stress, e.g., at times when frost is present.

High Heat

Transgenic plants expressing a recombinant MAPKKK kinase domain are also assayed in artificial environments or in the field in order to demonstrate that the transgene confers resistance or tolerance to heat.

Oxidative Stress

Oxidative stress is a major cause of damage in plants exposed to stressful environmental conditions. Oxidative stress results from the cellular damage caused by reactive oxygen species that are generated in cells. These reactive oxygen molecules can damage membranes, proteins, and nucleic acids. Transgenic plants that express a recombinant kinase domain of a MAPKKK are analyzed for the ability to improve resistance to oxidative stress.

Chemical Stress

Transgenic plants expressing a recombinant kinase domain of a MAPKKK are assayed in artificial environments or in the field to demonstrate that the transgene confers resistance or tolerance to chemicals (e.g., herbicides, ozone, or pollutants) or metals (e.g., copper or zinc). Transgenic plants having an increased ability to grow in the presence of higher concentrations of the toxic compound, as compared to non-transgenic plants, are useful in the invention.

Engineering Transgenic Plants Having Increased Yield/Productivity

To test the ability of the genes and domains described herein to improve crop yield or productivity, seeds of transgenic plants expressing a recombinant kinase domain of a MAPKKK are planted in test plots, and their agronomic performance is compared to standard plants using techniques familiar to those of skill in the art. Optionally included in this comparison are plants of similar genetic background without the transgene. A yield benefit is observed and plants exhibiting the increased yield are advanced for commercialization.

In addition, transgenic plants expressing a recombinant kinase domain are field tested for agronomic performance under conditions, including, but not limited to, limited or inadequate water availability. When compared to nontransgenic plants, transgenic plants expressing the kinase domain exhibit higher yield than their non-transgenic counterparts under non-optimal growing conditions.

Engineering Transgenic Plants Having Altered Seed Development

Constitutive expression of a recombinant kinase domain of a MAPKKK is useful for the production of seedless fruits and vegetables (e.g. watermelon, grape, tomato, and eggplant). Alternatively, by using a cycling promoter (e.g., a cyclin A or cyclin D promoter), expression of a recombinant MAPKKK or its kinase domain can be used to promote cell division resulting in the production of larger seeds. Furthermore, expression of a kinase domain under the control of an embryo- or endosperm-specific promoter can be used to control embryo or endosperm development (for example, the production of more endosperm and little or no embryo; or for the production of a larger embryo, but little or no endosperm).

Use

The invention described herein is useful for a variety of agricultural and

commercial purposes including, but not limited to, improving resistance or tolerance to stress, increasing crop yields, improving crop and ornamental quality, and reducing agricultural production costs. In particular, ectopic expression of a kinase domain of a MAPKKK polypeptide (or a MAPKKK polypeptide or a kinase domain-containing fragment thereof) (Figs. 11, 12, 13, 14, 15, and 16; SEQ ID NOS: 7-22) in a plant cell provides resistance to environmental stress and can be used to protect plants from adverse conditions that reduces plant productivity and viability. The invention therefore provides resistance to a variety of adverse environmental stresses to plants, especially crop plants, most especially crop plants such as tomato, potato, cotton, pepper, maize, wheat, rice, and legumes such as soybean, or any crop plant that is susceptible to an adverse stress. For example, transgenic maize and soybean may be genetically engineered to express a kinase domain of a MAPKKK (e.g., NPK1 or an ANP such as ANP1, ANP2, or ANP3) according to standard methods, such as those described in Adams et al. (U.S. Pat. 5,550,318) and Collins et al. (U.S. Pat. 5,024,944). Methods for transforming wheat with such genes are described in Fry et al. (U.S. Pat. 5,631,152).

Other Embodiments

The invention further includes the use of analogs of any naturally-occurring MAPKKK polypeptide. Analogs can differ from the naturally-occurring kinase domain by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the invention will generally exhibit at least 40%, more preferably 50%, and most preferably 60% or even having 70%, 80%, or 90% identity with all or part of a naturally-occurring kinase domain amino acid sequence. The length of sequence comparison is at least 15 amino acid residues, preferably at least 25 amino acid residues, and more preferably more than 35 amino acid residues. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide

synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring kinase domain polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethyl methylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual* (2d ed.), CSH Press, 1989, or Ausubel et al., *supra*). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids.

In addition, the invention also includes kinase domain fragments. As used herein, the term "fragment," means at least 50 contiguous amino acids, preferably at least 130 contiguous amino acids, more preferably at least 160 contiguous amino acids, and most preferably at least 190 to 230 or more contiguous amino acids. Fragments of kinase domain polypeptides can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events). In preferred embodiments, a kinase domain fragment (e.g., a fragment of NPK1, ANP1, ANP2, or ANP3) is capable of activating the transcription of a stress protective gene, repressing the transcription of an early auxin response gene transcription, or altering seed development. Methods for evaluating such activity are described herein.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.